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(54) Title: MATERIALS COMPRISING AND METHODS OF PREPARATION AND USE FOR RIBOSOME-INACTIVATING PROTEINS (57) Abstract <p>The present invention provides purified and isolated polynucleotides encoding Type I ribosome-inactivating proteins (RIPs) and analogs of the RIPs having a cysteine available for disulfide bonding to targeting molecules. Vectors comprising the polynucleotides and host cells transformed with the vectors are also provided. The RIPs and RIP analogs are particularly suited for use as components of cytotoxic therapeutic agents of the invention which include gene fusion products and immunoconjugates. Cytotoxic therapeutic agents or immunotoxins according to the present invention may be used to selectively eliminate any cell type to which the RIP component is targeted by the specific binding capacity of the second component of the agent, and are suited for treatment of diseases where the elimination of a particular cell type is a goal, such as autoimmune disease, cancer and graft-versus-host disease.</p>		

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MATERIALS COMPRISING AND METHODS OF PREPARATION AND USE FOR RIBOSOME-INACTIVATING PROTEINS

5 This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 07/901,707 filed June 19, 1992, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/787,567 filed November 4, 1991, now abandoned.

BACKGROUND

10 The present invention relates, in general, to materials useful as components of cytotoxic therapeutic agents. More particularly, the invention relates to polynucleotides encoding ribosome-inactivating proteins, to polynucleotides encoding analogs of ribosome-inactivating proteins specifically modified for conjugation to targeting molecules and to gene fusions of polynucleotides encoding ribosome-inactivating proteins to polynucleotides encoding targeting molecules.

15 Ribosome-inactivating proteins (RIPs) comprise a class of proteins which is ubiquitous in higher plants. RIPs have also been isolated from bacteria. RIPs are potent inhibitors of eukaryotic protein synthesis. The N-glycosidic bond of a specific adenine base is hydrolytically cleaved by RIPs in a highly conserved loop region of the 28S rRNA of eukaryotic ribosomes thereby inactivating translation.

20 Stirpe et al., FEBS Lett., 195(1,2), 1-8 (1986) groups plant RIPs into two types. Type I proteins each consist of a single peptide chain having ribosome-inactivating activity, while Type II proteins each consist of an A-chain, essentially equivalent to a Type I protein, disulfide-linked to a B-chain having cell-binding properties. Gelonin, dodecandrin, tricosanthin, tricokirin, bryodin, Mirabilis antiviral protein (MAP), barley ribosome-inactivating protein (BRIP), pokeweed antiviral
25 proteins (PAPs), saporins, luffins and momordins are examples of Type I RIPs, while ricin and abrin are examples of Type II RIPs. Amino acid sequence information is reported for various ribosome-inactivating proteins. It appears that at least the tertiary structure of active sites is conserved among Type I RIPs, bacterial RIPs and

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A-chains of Type II RIPs and, in many cases, primary structure homology is also found. Ready et al., J. Biol. Chem., 259(24), 15252-15256 (1984) and other reports suggest that the two types of RIPs are evolutionarily related.

Separated from their natural environment, Type I plant ribosome-inactivating proteins may be particularly suited for use as components of cytotoxic therapeutic agents. A RIP may be conjugated to a targeting agent that will deliver the RIP to a particular cell type in vivo in order to selectively kill those cells. Typically, the targeting agent (e.g., an antibody) is linked to the toxin by a disulfide bond which is reduced in vivo allowing the protein toxin to separate from the delivery antibody and become active intracellularly. Another strategy for producing a cytotoxic agent is to express a gene encoding a RIP fused to a gene encoding a targeting moiety. The resulting protein product is a single polypeptide containing an RIP linked to, for example, at least one chain of an antibody. A variety of gene fusion products including protein toxin sequences are discussed in a recent review by Pastan et al., Science, 254, 1173-1177 (1991).

Because some RIPs, such as the Type I RIP gelonin, are only available from scarce plant materials, it is desirable to clone the genes encoding the RIPs to enable recombinant production of the proteins. It is also desirable to develop analogs of the natural proteins which may be easily conjugated to targeting molecules while retaining their natural biological activity because most Type I RIPs have no natural sites (i.e. available cysteine residues) for conjugation to targeting agents. Alternatively, it is desirable to develop gene fusion products including Type I RIPs as a toxic moiety and antibody substances as a targeting moiety.

There thus exists a need in the art for cloned genes encoding Type I RIPs, for analogs of Type I RIPs which may be easily conjugated to targeting molecules and for gene fusion products comprising Type I RIPs.

SUMMARY OF THE INVENTION

The present invention provides purified and isolated polynucleotides encoding Type I RIPs, Type I RIPs having a cysteine available for disulfide bonding to targeting molecules and fusion products including Type I RIPs. Vectors comprising the polynucleotides and host cells transformed with the vectors are also provided.

A purified and isolated polynucleotide encoding natural sequence gelonin, and a host cell including a vector encoding gelonin of the type deposited as ATCC Accession No. 68721 are provided. Further provided are a purified and isolated polynucleotide encoding natural sequence barley ribosome-inactivating protein and a purified and isolated polynucleotide encoding momordin II.

Analog of a Type I plant RIP are defined herein as non-naturally occurring polypeptides that share the ribosome-inactivating activity of the natural protein but that differ in amino acid sequence from the natural protein. Preferred analogs according to the present invention are analogs of Type I plant RIPs each having a cysteine available for disulfide bonding located at a position in its amino acid sequence from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. Other preferred analogs according to the invention are Type I RIPs each having a cysteine available for disulfide bonding at a position in the analog that is on the surface of the protein in its natural conformation and that does not impair native folding or biological activity of the ribosome-inactivating protein. Analog of bacterial RIPs are also contemplated by the present invention.

The present invention provides an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog corresponding to position 259 in SEQ ID No: 1 or at a position in the amino acid

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may encode an analog of gelonin, preferably an analog wherein the cysteine is at a position in the amino acid sequence of the analog from position 244 to the carboxyl terminal position of the analog, more preferably wherein the cysteine is at a position in the analog from position 247 to the carboxyl terminal position of the analog, and most preferably the cysteine is at position 244, at position 247 or at position 248 of the amino acid sequence of the analog. It is preferred that a polynucleotide according to the present invention encode a gelonin analog wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues.

A polynucleotide according to the present invention may encode an analog of barley ribosome-inactivating protein, preferably an analog wherein the cysteine is at a position in the analog from position 256 to the carboxyl terminal position of the analog, more preferably wherein the cysteine is at a position in the analog from position 260 to the carboxyl terminal position of the analog, and most preferably wherein the cysteine is at position 256, at position 270 or at position 277 of the amino acid sequence of the analog.

A polynucleotide according to the present invention may encode an analog of mormordin II.

The present invention provides a vector including a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at a amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

The present invention further provides a host cell including a DNA vector encoding an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding

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to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. In such a host cell the vector may encode an analog of gelonin, especially an analog wherein the cysteine is at position 247 of the amino acid sequence of the analog, such as in the host cell deposited as ATCC Accession No. 69009.

5 A host cell according to the present invention may include a vector encoding barley ribosome-inactivating protein, especially a host cell wherein the cysteine is at position 277 of the amino acid sequence of the analog such as in the host cell deposited as ATCC Accession No. 68722.

10 The present invention also provides an agent toxic to a cell including an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, which cysteine is at an amino acid position in the analog corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located in the amino acid sequence of the analog from
15 the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. The agent may include an analog of gelonin, preferably an analog wherein the cysteine is at a position in the analog from position 247 to the carboxyl terminal position of the analog, and more preferably wherein the cysteine is at position 247 or 248 of the amino acid sequence of analog. An agent including
20 an analog wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues is preferred.

An agent according to the present invention may include an analog of barley ribosome-inactivating protein, preferably an analog wherein the cysteine is at a position in the analog from position 260 to the carboxyl terminal position of the
25 analog, more preferably wherein the cysteine is at a position in the analog from position 270 to the carboxyl terminal position of the analog, and most preferably wherein the cysteine is at position 256, at position 270 or at position 277 of the amino acid sequence of the analog.

30 An agent according to the present invention may include an analog of momordin II.

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The present invention provides an agent wherein the Type I ribosome-inactivating protein is linked to an antibody, particularly to an H65 antibody or to an antibody fragment, more particularly to an antibody fragment selected from the group consisting of chimeric and human engineered antibody fragments, and most particularly to a Fab antibody fragment, a Fab' antibody fragment or a F(ab')₂ antibody fragment. It is highly preferred that an agent according to the present invention include a chimeric or human engineered antibody fragment selected from the group consisting of a Fab antibody fragment, a Fab' antibody fragment and a F(ab')₂ antibody fragment.

A method according to the present invention for preparing an analog of a Type I ribosome-inactivating protein includes the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted (e.g., by site-directed mutagenesis of the natural DNA sequence encoding the RIP or by chemical synthesis of a DAN sequence encoding the RIP analog) at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein, which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

A product according to the present invention may be a product of a method including the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein, which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

The present invention provides a method for preparing an agent toxic to a cell including the step of linking an analog of a Type I ribosome-inactivating protein through a cysteine to a molecule which specifically binds to the cell, which

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analog has the cysteine at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

According to the present invention, a method for treating a disease in which elimination of particular cells is a goal may include the step of administering to a patient having the disease a therapeutically effective amount of an agent toxic to the cells including an analog of a Type I ribosome-inactivating protein linked through a cysteine to a molecule which specifically binds to the cell, the analog having the cysteine at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and the cysteine being located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

The present invention also provides an analog of a Type I ribosome-inactivating protein, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

Such an analog may be an analog wherein the Type I ribosome inactivating protein is gelonin, and is preferably an analog of gelonin wherein the cysteine is at position 10 of the amino acid sequence of the analog as encoded in a vector in a host cell deposited as ATCC Accession No. 69008. Other such gelonin analogs include those wherein the cysteine is at a position 60, 103, 146, 184 or 215 in the amino acid sequence of the gelonin analog. It is preferred that the gelonin cysteine residues at positions 44 and 50 be replaced with alanine residues in these analogs.

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The present invention further provides an analog of a Type I ribosome-inactivating protein wherein the analog includes only a single cysteine. Such an analog may be an analog of gelonin and is preferably an analog wherein the single cysteine is at position 10, position 44, position 50 or position 247 in the amino acid sequence of the analog, but the cysteine may be located at other positions defined by the invention as well.

The present invention provides a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

According to the present invention, a method for preparing an analog of a Type I ribosome-inactivating protein may include the step of expressing in suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for disulfide bonding in the Type I ribosome-inactivating protein, the cysteine is located at a position corresponding to an amino acid position on the surface of ricin A-chain in its natural conformation and which analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

The present invention provides an agent toxic to a cell including an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the

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analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

5 A method according to the present invention for preparing an agent toxic to a cell may include the step of linking an analog of a Type I ribosome-inactivating protein through a cysteine to a molecule which specifically binds to the cell, which analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and which analog retains ribosome-inactivating activity of the Type I
10 ribosome-inactivating protein.

A method according to the present invention for treating a disease in which elimination of particular cells is a goal includes the step of administering to a patient having the disease a therapeutically effective amount of an agent toxic to the
15 cells wherein the agent includes an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, which analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and which analog retains ribosome-inactivating activity of the Type I
20 ribosome-inactivating protein.

The RIP analogs are particularly suited for use as components of cytotoxic therapeutic agents. Cytotoxic agents according to the present invention may
25 be used in vivo to selectively eliminate any cell type to which the RIP component is targeted by the specific binding capacity of the second component. To form cytotoxic agents RIP analogs may be conjugated to monoclonal antibodies, including chimeric and CDR-grafted antibodies, and antibody domains/fragments (e.g., Fab, Fab', F(ab')₂, single chain antibodies, and Fv or single variable domains) as well as
30 conjugation to monoclonal antibodies genetically engineered to include free cysteine

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residues are within the scope of the present invention. Examples of Fab' and F(ab')₂ fragments useful in the present invention are described in co-pending, co-owned U.S. Patent Application Serial No. 07/714,175, filed June 14, 1991 and in International Publication No. WO 89/00999 published on February 9, 1989, which are
5 incorporated by reference herein. RIPs according to the present invention may also be conjugated to targeting agents other than antibodies, for example, lectins which bind to cells having particular surface carbohydrates, or hormones, lymphokines, growth factors or other polypeptides which bind specifically to cells having particular receptors. Immunoconjugates including RIPs may be described as immunotoxins.
10 An immunotoxin may also consist of a fusion protein rather than an immunoconjugate.

The present invention provides gene fusions of an antigen-binding portion of an antibody (e.g., an antibody light chain or truncated heavy chain, or a single chain antibody) or any targeting agent listed in the foregoing paragraph, linked
15 to a Type I RIP. The gene fusions may include an RIP gene linked either at the 5' or the 3' end of an antibody gene. A DNA linker encoding a peptide segment may or may not be inserted between the toxin and the antibody gene. Preferably, the linker encodes a segment of the *E. coli* shiga-like toxin which contains two cysteine residues participating in a disulfide bond and forming a loop that includes a protease
20 sensitive amino acid sequence (e.g., SEQ ID NO: 58), or a segment of rabbit muscle aldolase which contains several potential cathepsin cleavage sites (e.g., SEQ ID NO: 59). The Type I RIP portion of the fused genes preferably encodes gelonin, BRIP or momordin II. Also preferably, the antibody portion of the fused genes comprises sequences encoding one of the chains of an antibody Fab fragment (i.e., kappa or Fd)
25 and the fused gene is co-expressed in a host cell with the other Fab gene, or the antibody portion comprises sequences encoding a single chain antibody.

The present invention also provides a method for purifying an immunotoxin comprising a ribosome-inactivating protein and a portion of an antibody including the steps of passing a solution containing the immunotoxin through an anion
30 exchange column; applying the flow-through to a protein G column; and eluting the

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immunotoxin from the protein G column. The method may further comprise the steps of introducing the flow-through of the anion exchange column into a cation exchange column; exposing the cation exchange column to an eluent effective to elute said protein; and then applying the flow-through to a protein G column, rather than
5 applying the anion exchange column flow-through directly to a protein G column.

Immunotoxins according to the present invention including cytotoxic agents and fusion proteins are suited for treatment of diseases where the elimination of a particular cell type is a goal, such as autoimmune disease, cancer and graft-versus-host disease. The immunotoxins are also suited for use in causing
10 immunosuppression and in treatment of infections by viruses such as the Human Immunodeficiency Virus.

Specifically illustrating polynucleotide sequences according to the present invention are the inserts in the plasmid pING3731 in E. coli MC1061 (designated strain G274) and in the plasmid pING3803 in E. coli E104 (designated
15 strain G275), both deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, on October 2, 1991, and assigned ATCC Accession Nos. 68721 and 68722, respectively. Additional polynucleotide sequences illustrating the invention are the inserts in the plasmid pING3746 in E. coli E104 (designated strain G277) and in the plasmid pING3737 in E. coli E104
20 (designated strain G276), which were both deposited with the ATCC on June 9, 1992, and were respectively assigned Accession Nos. 69008 and 69009. Still other polynucleotide sequences illustrating the invention are the inserts in the plasmid pING3747 in E. coli E104 (designated strain G278), in the plasmid pING3754 in E. coli E104 (designated strain G279), in the plasmid pING3758 in E. coli E104
25 (designated strain G280) and in the plasmid pING3759 in E. coli E104 (designated strain G281), which plasmids were all deposited with the ATCC on October 27, 1992 and were assigned ATCC Accession Nos. 69101, 69102, 69103 and 69104, respectively.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (RTA) (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein gelonin (SEQ ID NO: 2), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 2 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein BRIP (SEQ ID NO: 3), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 3 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein momordin II (MOMOI) (SEQ ID NO: 4), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 4 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein luffin (SEQ ID NO: 5), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 5 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein atrichosanthin (TRICHO) (SEQ ID NO: 6), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 6 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein momordin I (MOMOI) (SEQ ID NO: 7), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 7 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I

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Type I RIP analogs of the present invention offer distinct advantages over the natural proteins for use as components of immunotoxins. Chemical treatment to introduce free sulfhydryl groups in the natural proteins lacking free cysteines typically involves the non-selective modification of amino acid side chains. This non-selectivity often results in antibodies conjugated to different sites on different RIP molecules (i.e., a heterogeneous population of conjugates) and also in

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a decrease in RIP activity if antibodies are conjugated in or near important regions of the RIP (e.g., the active site or regions involved in translocation across cell membranes). In contrast, RIP analogs according to the present invention can be conjugated to a single antibody through a disulfide bond to a specific residue of the analog resulting in reduced batch to batch variation of the immunoconjugates and, in some cases, immunoconjugates with enhanced properties (e.g., greater cytotoxicity or solubility).

Type I plant RIPs, as well as bacterial RIPs such as shiga and shiga-like toxin A-chains, are homologous to the ricin A-chain and are useful in the present invention.

Type I RIPs may be defined and sites for substitution of a cysteine in a RIP may be identified by comparing the primary amino acid sequence of the RIP to the natural ricin A-chain amino acid sequence, the tertiary structure of which has been described in Katzin et al., Proteins, 10, 251-259 (1991), which is incorporated by reference herein.

Amino acid sequence alignment defines Type I RIPs in that the ricin A-chain and the Type I plant RIPs have nine invariant amino acids in common. Based on the ricin sequence the invariant amino acids are tyrosine₂₁, arginine₂₉, tyrosine₄₀, tyrosine₁₂₃, leucine₁₄₄, glutamic acid₁₇₇, alanine₁₇₈, arginine₁₈₀, and tryptophan₂₁₁. The ricin A-chain may be used as a model for the three-dimensional structure of Type I RIPs. A protein lacking a cysteine available for conjugation while having ribosome-inactivating activity and having the nine invariant amino acids when its primary sequence is compared to the primary sequence of the ricin A-chain [according to the alignment algorithm of Myers et al., CABIOS COMMUNICATIONS, 4(1), 11-17 (1988), implemented by the PC/GENE program PALIGN (Intelligenetics, Inc., Mountain View, California) and utilizing the Dayhoff Mutation Data Matrix (MDM-78) as described in Schwartz et al., pp. 353-358 in Atlas of Protein Sequence and Structure, 5 Supp. 3, National Biomedical Research Foundation, Washington, D.C. (1978)] is defined as a Type I RIP herein and is expected to be useful in the present invention. "Corresponding" refers herein to

amino acid positions that align when two amino acid sequences are compared by the strategy of Myers et al., *supra*.

The primary amino acid sequences of the Type I RIPs gelonin, BRIP, momordin II, luffin [see Islam et al., *Agricultural Biological Chem.*, 54(5), 1343-1345 (199)], atrichosanthin [see Chow et al., *J. Biol. Chem.*, 265, 8670-8674 (1990)], momordin I [see Ho et al., *BBA*, 1088, 311-314 (1991)], *Mirabilis* anti-viral protein [see Habuka et al., *J. Biol. Chem.*, 264(12), 6629-6637 (1989)], pokeweed antiviral protein isolated from seeds [see Kung et al., *Agric. Biol. Chem.*, 54(12), 3301-3318 (1990)] and saporin [see Benatti et al., *Eur. J. Biochem.*, 183, 465-470 (1989)] are individually aligned with the primary sequence of the ricin A-chain [see Halling et al., *Nucleic Acids Res.*, 13, 8019-8033 (1985)] in FIGs 1-9, respectively, according to the algorithm of Myers et al., *supra*, as specified above.

FIGs 1-9 may be utilized to predict the amino acid positions of the Type I RIPs where cysteine residues may be substituted. Preferred amino acids for cysteine substitution are on the surface of the molecule and include any solvent accessible amino acids that will not interfere with proper folding of the protein if replaced with a cysteine. A region of the ricin A-chain comprising such amino acids is the carboxyl terminal region. Amino acids that should be avoided for replacement are those critical for proper protein folding, such as proline, and those that are solvent inaccessible. Also to be avoided are the nine amino acids invariant among RIPs, and the amino acids in or near regions comprising the active site of ricin A-chain as depicted in Figure 6 of Katzin et al., *supra*.

Therefore, a preferred region of substitution for Type I RIPs is their carboxyl terminal region which is solvent accessible and corresponds to the carboxyl terminal region where Type II RIP A-chains and B-chains are naturally linked by a disulfide bond. As shown in the examples, a cysteine may be substituted in positions in the amino acid sequence of a Type I RIP from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said Type I RIP, resulting in RIP analogs which retain enzymatic activity and gain disulfide cross-

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linking capability. One preferred cysteine substitution position is near the position which corresponds to the cysteine at position 259 in the ricin A-chain.

Immunotoxins specifically illustrating the present invention including cytotoxic agents and gene fusion products are particularly suited for use in treatment of human autoimmune disease where T-cell function is implicated. Treatment of autoimmune diseases with immunotoxins is described in co-owned U.S. Patent Application Serial No. 07/306,433 filed on September 13, 1991 and in International Publication No. WO89/06968 published August 10, 1989, which are incorporated by reference herein. Examples of autoimmune diseases are systemic lupus erythematosus, scleroderma diseases (including lichen sclerosus, morphea and lichen planus), rheumatoid arthritis, chronic thyroiditis, pemphigus vulgaris, diabetes mellitus type 1, progressive systemic sclerosis, aplastic anemia, myasthenia gravis, myositis, Sjogrens disease, Crohn's disease, ulcerative colitis, and primary biliary cirrhosis. Autoimmunity is also implicated in multiple sclerosis, uveitis, psoriasis and Meniere's disease. A general description of various autoimmune diseases may be found in Rose and Mackey, Eds., The Autoimmune Diseases, Academic Press (1985).

The immunotoxins may be administered to a patient either singly or in a cocktail containing two or more immunotoxins, other therapeutic agents, compositions, or the like, including, but not limited to, immunosuppressive agents, tolerance-inducing agents, potentiators and side-effect relieving agents. Particularly preferred are immunosuppressive agents useful in suppressing allergic reactions of a host. Preferred immunosuppressive agents include prednisone, prednisolone, DECADRON (Merck, Sharp & Dohme, West Point, Pennsylvania), cyclophosphamide, cyclosporine, 6-mercaptopurine, methotrexate, azathioprine and i.v. gamma globulin or their combination. Preferred potentiators include monensin, ammonium chloride, perhexiline, verapamil, amantadine and chloroquine. All of these agents are administered in generally-accepted efficacious dose ranges such as those disclosed in the Physician's Desk Reference, 41st Ed., Publisher Edward R. Barnhart, New Jersey (1987). Patent Cooperation Treaty (PCT) patent application

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WO 89/069767 published on August 10, 1989, discloses administration of an immunotoxin as an immunosuppressive agent and is incorporated by reference herein.

Anti-T cell immunotoxins may be formulated into either an injectable or topical preparation. Parenteral formulations are known and are suitable for use in the invention, preferably for intramuscular or intravenous administration. The formulations containing therapeutically-effective amounts of anti-T cell immunotoxins are either sterile liquid solutions, liquid suspensions or lyophilized versions, and optionally contain stabilizers or excipients. Lyophilized compositions are reconstituted with suitable diluents, e.g., water for injection, saline, 0.3% glycine and the like, at a level of about from 0.01 mg/kg of host body weight to 10 mg/kg where the biological activity is less than or equal to 20 ng/ml when measured in a reticulocyte lysate assay. Typically, the pharmaceutical compositions containing anti-T cell immunotoxins will be administered in a therapeutically effective dose in a range of from about 0.01 mg/kg to about 5 mg/kg of the patient. A preferred, therapeutically effective dose of the pharmaceutical composition containing anti-T cell immunotoxin will be in a range of from about 0.01 mg/kg to about 0.5 mg/kg body weight of the patient administered over several days to two weeks by daily intravenous infusion, each given over a one hour period, in a sequential patient dose-escalation regimen.

Anti-T cell immunotoxin is formulated into topical preparations for local therapy by including a therapeutically effective concentration of anti-T cell immunotoxin in a dermatological vehicle. The amount of anti-T cell immunotoxin to be administered, and the anti-T cell immunotoxin concentration in the topical formulations, depends upon the vehicle selected, the clinical condition of the patient, the systemic toxicity and the stability of the anti-T cell immunotoxin in the formulation. Thus, a physician knows to employ the appropriate preparation containing the appropriate concentration of anti-T cell immunotoxin in the formulation, as well as the appropriate amount of formulation to administer depending upon clinical experience with the patient in question or with similar patents. The concentration of anti-T cell immunotoxin for topical formulations is in the range of

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greater than from about 0.1 mg/ml to about 25 mg/ml. Typically, the concentration of anti-T cell immunotoxin for topical formulations is in the range of greater than from about 1 mg/ml to about 20 mg/ml. Solid dispersions of anti-T cell immunotoxin as well as solubilized preparations can be used. Thus, the precise concentration to be used in the vehicle is subject to modest experimental manipulation in order to optimize the therapeutic response. Greater than about 10 mg anti-T cell immunotoxin/100 grams of vehicle may be useful with 1% w/w hydrogel vehicles in the treatment of skin inflammation. Suitable vehicles, in addition to gels, are oil-in-water or water-in-oil emulsions using mineral oils, petroleum and the like.

Anti-T cell immunotoxin is optionally administered topically by the use of a transdermal therapeutic system [Barry, Dermatological Formulations, p. 181 (1983) and literature cited therein]. While such topical delivery systems have been designed for transdermal administration of low molecular weight drugs, they are capable of percutaneous delivery. They may be readily adapted to administration of anti-T cell immunotoxin or derivatives thereof and associated therapeutic proteins by appropriate selection of the rate-controlling microporous membrane.

Topical preparations of anti-T cell immunotoxin either for systemic or local delivery may be employed and may contain excipients as described above for parenteral administration and other excipients used in a topical preparation such as cosolvents, surfactants, oils, humectants, emollients, preservatives, stabilizers and antioxidants. Any pharmacologically-acceptable buffer may be used, e.g., Tris or phosphate buffers. The topical formulations may also optionally include one or more agents variously termed enhancers, surfactants, accelerants, adsorption promoters or penetration enhancers, such as an agent for enhancing percutaneous penetration of the anti-T cell immunotoxin or other agents. Such agents should desirably possess some or all of the following features as would be known to the ordinarily skilled artisan: pharmacological inertness, non-promotive of body fluid or electrolyte loss, compatible with anti-T cell immunotoxin (non-inactivating), and capable of formulation into creams, gels or other topical delivery systems as desired.

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Anti-T cell immunotoxin may also be administered by aerosol to achieve localized delivery to the lungs. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing immunotoxin. Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of anti-T cell immunotoxin together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary depending upon the requirements for the particular anti-T cell immunotoxin, but typically include: nonionic surfactants (Tweens, Pluronic, or polyethylene glycol); innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin; amino acids such as glycine; and buffers, salts, sugars or sugar alcohols. The formulations may also include mucolytic agents as well as bronchodilating agents. The formulations are sterile. Aerosols generally are prepared from isotonic solutions. The particles optionally include normal lung surfactants.

Aerosols may be formed of the particles in aqueous or nonaqueous (e.g., fluorocarbon propellant) suspension. Such particles include, for example, intramolecular aggregates of anti-T cell immunotoxin or derivatives thereof or liposomal or microcapsular-entrapped anti-T cell immunotoxin or derivatives thereof. The aerosols should be free of lung irritants, i.e., substances which cause acute bronchoconstriction, coughing, pulmonary edema or tissue destruction. However, nonirritating absorption-enhancing agents are suitable for use herein. Sonic nebulizers are preferably used in preparing aerosols. Sonic nebulizers minimize exposing the anti-T cell immunotoxin or derivatives thereof to shear, which can result in degradation of anti-T cell immunotoxin.

Anti-T cell immunotoxin may be administered systemically, rather than topically, by injection intramuscularly, subcutaneously, intrathecally or intraperitoneally or into vascular spaces, particularly into the joints, e.g., intraarticular injection at a dosage of greater than about 1 μ g/cc joint fluid/day. The dose will be dependent upon the properties of the anti-T cell immunotoxin employed, e.g., its activity and biological half-life, the concentration of anti-T cell immunotoxin in the formulation, the site and rate of dosage, the clinical tolerance of the patient

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involved, the autoimmune disease afflicting the patient and the like, as is well within the skill of the physician.

The anti-T cell immunotoxins of the present invention may be administered in solution. The pH of the solution should be in the range of pH 5 to 9.5, preferably pH 6.5 to 7.5. The anti-T cell immunotoxin or derivatives thereof should be in a solution having a suitable pharmaceutically-acceptable buffer such as phosphate, Tris(hydroxymethyl)aminomethane-HCl or citrate and the like. Buffer concentrations should be in the range of 1 to 100 mM. The solution of anti-T cell immunotoxin may also contain a salt, such as sodium chloride or potassium chloride in a concentration of 50 to 150 mM. An effective amount of a stabilizing agent such as an albumin, a globulin, a gelatin, a protamine or a salt of protamine may also be included, and may be added to a solution containing anti-T cell immunotoxin or to the composition from which the solution is prepared.

Systemic administration of anti-T cell immunotoxin is made daily and is generally by intramuscular injection, although intravascular infusion is acceptable. Administration may also be intranasal or by other nonparenteral routes. Anti-T cell immunotoxin may also be administered via microspheres, liposomes or other microparticulate delivery systems placed in certain tissues including blood. Topical preparations are applied daily directly to the skin or mucosa and are then preferably occluded, i.e., protected by overlaying a bandage, polyolefin film or other barrier impermeable to the topical preparation.

The following examples illustrate practice of the invention but are not to be construed as limiting the invention. Example 1 is a description of the cloning of a cDNA encoding the Type I RIP gelonin. Example 2 describes the construction of recombinant expression vectors containing the gelonin gene. Described in Example 3 are constructions of various analogs of gelonin having a single cysteine available for disulfide bonding. Example 4 describes the testing of recombinant gelonin and the gelonin analogs for the capacity to inhibit protein synthesis in a reticulocyte lysate assay. Example 5 presents descriptions of the preparation of various gelonin immunoconjugates. Example 6 describes of the testing of the

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immunoconjugates for the capacity to act as cytotoxic agents in a whole cell kill assay. Example 7 presents solubility and stability characteristics of the immunoconjugates. Examples 8 presents results of in vivo pharmacokinetic and immunogenicity studies of the gelonin immunoconjugates and Example 9 presents results of testing of the immunoconjugates for the capacity to deplete human T cells in a human peripheral blood lymphocyte-reconstituted, severe combined immunodeficient mouse model. Described in Example 10 are various gene fusions of gelonin DNA sequences and sequences encoding antibody fragments. Expression of products of the gene fusions products and testing of the products in the reticulocyte and whole cell kill assays are described in Example 11. Example 12 is a description of the construction of gelonin gene fusions to single chain antibodies. Example 13 describes the cloning of a cDNA encoding the Type I RIP BRIP, construction of expression vectors containing the BRIP gene, production of BRIP analogs having a single cysteine available for disulfide bonding, testing of the analogs in the reticulocyte lysate assay, and construction of the BRIP immunoconjugates and testing of their activity in the whole cell kill assay. Example 14 describes the cloning of a cDNA encoding momordin II and construction of expression vectors containing the momordin II gene.

Example 1

The cloning of the gelonin gene according to the present invention obviates the requirement of purifying the RIP gene product from its relatively scarce natural source, G. multiflorum seeds, and allows development of gelonin analogs conjugatable to antibodies without prior chemical derivatization and development of gelonin gene fusion products. One formidable hurdle in the cloning of the gene was that the available Gelonium seeds are old and inviable, making preparation of intact messenger RNA from the seeds impossible. Cloning the gene from cDNA prepared from messenger RNA was thus impractical and total RNA was utilized to generate cDNA. Using total RNA to make cDNA under normal circumstances, i.e., when

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mRNA may be utilized, is not desirable because total RNA typically comprises about 95% ribosomal RNA.

Preparation of RNA from *G. multiflorum* Seeds

5 Total RNA was prepared from *Gelonium* seeds (Dr. Michael Rosenblum, M.D. Anderson Cancer Center, Houston, Texas) by a modification of the procedure for preparation of plant RNA described in Ausubel et al., eds., Current
10 Protocols in Molecular Biology, Wiley & Sons, 1989. Briefly, 4.0 grams of seeds were ground to a fine powder in a pre-cooled (-70°C) mortar and pestle with liquid N₂. The powder was added to 25 ml Grinding buffer (0.18M Tris, 0.09M LiCl, 4.5mM EDTA, 1% SDS, pH 8.2) along with 8.5 ml of phenol equilibrated with TLE
15 (0.2M Tris, 0.1M LiCl, 5mM EDTA pH8.2). The mixture was homogenized using a Polytron PT-1035 (#5 setting). 8.5 ml of chloroform was added, mixed and incubated at 50°C for 20 minutes. The mixture was centrifuged at 3K for 20 minutes in a rotor precooled to 4°C and the aqueous phase was transferred to a new tube. 8.5
20 ml of phenol was added followed by 8.5 ml of chloroform and the mixture was recentrifuged. This extraction was repeated 3 times. The RNA in the aqueous phase was then precipitated by adding 1/3 volume 8M LiCl, and incubated at 4°C for 16 hours. Next, the RNA was pelleted by centrifugation for 20 minutes at 4°C. The pellet was washed with 5 ml of 2M LiCl, recentrifuged and resuspended in 2 ml of water. The RNA was precipitated by addition of NaOAc to 0.3M and 2 volumes of ethanol. The RNA was stored in 70% ethanol at -70°C.

cDNA Preparation

cDNA was prepared from total *Gelonium* RNA by two similar methods.
25 The first method involved making a cDNA library in the bacterial expression plasmid pcDNAII using the Librarian II cDNA Library Construction System kit (Invitrogen). About 5 µg of total RNA was converted to first strand cDNA with a 1:1 mixture of random primers and oligo-dT. Second strand synthesis with DNA polymerase I was performed as described by the system manufacturer. Double stranded cDNA was
30 ligated to BstXI linkers and size fractionated. Pieces larger than about 500 bp were

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ligated into the expression vector provided in the kit. Individual vectors were introduced into *E. coli* either by transformation into high-efficiency competent cells or by electroporation into electrocompetent cells. Electroporation was performed with a BTX100 unit (BTX, San Diego, CA) in 0.56 μ Flatpack cells as recommended by BTX based on the method of Dower et al., *Nucleic Acids Res.*, 16, 6127-6145 (1988), at a voltage amplitude of 850 V and a pulse length of 5 mS. The resulting library consisted of approximately 150,000 colonies.

The second method involved generating cDNA using the RNA-PCR kit sold by Perkin-Elmer-Cetus. About 100 ng of total Gelonium RNA was used as template for cDNA synthesis.

Determination of the Gelonin Protein Sequence

The partial sequence of the native gelonin protein was determined by direct amino acid sequence analysis by automated Edman degradation as recommended by the manufacturer using an Applied Biosystems model 470A protein sequencer. Proteolytic peptide fragments of gelonin (isolated from the same batch of seeds as the total RNA) were sequenced.

Cloning of the Gelonin Gene

Three overlapping gelonin cDNA fragments were cloned and a composite gelonin gene was assembled from the three fragments.

(1) Cloning of the Fragment Encoding the Middle Amino Acids of Gelonin in Vector pING3823.

Degenerate DNA primers based on the gelonin partial amino acid sequences were used to amplify by PCR, segments of the cDNA generated with the Perkin-Elmer-Cetus kit. Six primers were designed based on regions of the gelonin amino acid sequence where degeneracy of the primers could be minimized. Appropriate pairs of primers were tested for amplification of a gelonin gene fragment. When products of the expected DNA size were identified as ethidium bromide-stained DNA bands on agarose gels, the DNA was treated with T4 DNA polymerase and then purified from an agarose gel. Only the primer pair consisting of primers designated gelo-7 and gelo-5 yielded a relatively pure product of the expected size. The

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sequences of degenerate primers gelo-7 and gelo-5 are set out below using IUPAC nucleotide symbols.

Gelo-7 (SEQ ID NO: 14)

5' TTYAARGAYGCNCCNGAYGCNGCNTAYGARGG 3'

Gelo-5 (SEQ ID NO: 15)

3' TTYTTYATRATRCANTGNCGNCANCTRGTYCA 5'

Primer gelo-7 corresponds to amino acids 87-97 of gelonin while primer gelo-5 corresponds to amino acids 226-236. The blunt-ended DNA fragment (corresponding to amino acids 87 to 236 of gelonin) generated with primers gelo-7 and gelo-5 was cloned into pUC18 (BRL, Gaithersburg, Maryland). The DNA sequence of the insert was determined, and the deduced amino acid sequence based on the resulting DNA sequence matched the experimentally determined gelonin amino acid sequence. The clone containing this gelonin segment is denoted pING3726.

The insert of clone pING3726 was labeled with ³²P and used as a probe to screen the 150,000-member Gelonium cDNA library. Only one clone hybridized to the library plated in duplicate. This clone was purified from the library and its DNA sequence was determined. The clone contains a fragment encoding 185 of the 270 amino acids of gelonin (residues 25-209) and is denoted pING3823.

(2) Cloning of the Fragment Encoding the N-terminal Amino Acids of Gelonin.

Based on the sequence determined for the gelonin gene segment in pING3726, exact oligonucleotide primers were designed as PCR amplification primers to be used in conjunction with a degenerate primer to amplify a 5' gelonin gene fragment and with a nonspecific primer to amplify a 3' gelonin gene fragment. cDNA generated using the Perkin-Elmer-Cetus RNA-PCR kit was amplified.

To amplify the 5'-end of the gelonin gene, PCR amplification with a degenerate primer gelo-1 and an exact primer gelo-10 was performed. The sequences of the primers are set out below.

Gelo-1 (SEQ ID NO: 16)

5' GGN YTNGAYACNGTNWSNTTYWSNACNAARGG 3'

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Gelo-10 (SEQ ID NO: 17)

3' TGTCTGAACCCGTAACCTGGTAA 5'

Primer gelo-1 corresponds to amino acids 1-11 of the gelonin gene while primer gelo-10 corresponds to amino acids 126-133. The product from the reaction was re-amplified with gelo-1 (SEQ ID NO: 16) and gelo-11 (an exact primer comprising sequences encoding amino acids 119-125 of gelonin) to confer specificity to the reaction product. The sequence of primer gelo-11 is listed below.

Gelo-11 (SEQ ID NO: 18)

3' CACTCTTCCGTATATCTCTCTGT 5'

Hybridization with an internal probe confirmed that the desired specific gelonin DNA fragment was amplified. This fragment was cloned into pUC18, and the vector generated was designated pING3727. The fragment was sequenced revealing that the region of the fragment (the first 27 nucleotides) corresponding to part of the degenerate primer gelo-1 could not be translated to yield the amino acid sequence upon which primer gelo-1 was originally based. This was not unexpected considering the degeneracy of the primer. The fragment was reamplified from the Gelonium cDNA with exact primers gelo-11 (SEQ ID NO: 18) and gelo-5' (which extends upstream of the 5' end of the gelonin gene in addition to encoding the first 16 amino acids of gelonin). The sequence of primer gelo-5' is set out below.

Gelo-5' (SEQ ID NO: 19)

5' TCAACCCGGGCTAGATACCGTGTCAT

TCTCAACCAAAGGTGCCACTTATATTA 3'

The resulting DNA fragment encodes the first 125 amino acids of gelonin. While the majority of the sequence is identical to the natural gelonin gene, the first 32 nucleotides of the DNA fragment may not be. For the purposes of this application this N-terminal fragment is referred to as fragment GEL1-125.

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(3) Cloning of the Fragment Encoding the C-terminal Amino Acids of Gelonin.

To amplify the 3'-end of the gelonin gene as well as 3' untranslated sequences, PCR amplification with exact primers gelo-9 and XE-dT was performed. The sequence of each of the primers is set out below.

Gelo-9 (SEQ ID NO: 20)

5' CTTCATTTTGGCGGCACGTATCC 3'

XE-dT (SEQ ID NO: 21)

3' TTTTTTTTTTTTTTTTTTTTAG

GGTGCATTTCGAACGTCGGAGCTC 5'

Primer gelo-9 corresponds to amino acids 107-113 of gelonin. Primer XE-dT consists of an 3' oligo-dT portion and a 5' portion containing the restriction sites HindIII and XhoI, and will prime any poly A-containing cDNA. The reaction product was reamplified with exact primers gelo-8 and XE. The sequences of primers gelo-8 and XE are set out below.

Gelo-8 (SEQ ID NO: 22)

5' CTCGCTGGAAGGTGAGAA 3'

XE (SEQ ID NO: 23)

3' AGGGTGCATTTCGAACGTCGGAGCTC 5'

Primer gelo-8 consists of sequences encoding amino acids 115-120 of gelonin while the primer XE corresponds to the 5' portion of the XE-dT primer which contains HindIII and XhoI restriction sites. Hybridization with internal probes confirmed that the desired gelonin gene fragment was amplified. The fragment was cloned into pUC18 by two different methods. First, it was cloned as a blunt-ended fragment into the SmaI site of pUC18 (the resulting vector was designated pING3728) and, second, it was cloned as an EcoRI to HindIII fragment into pUC18 (this vector was designated pING3729). Both vector inserts were sequenced. The insert of pING3728 encodes amino acids 114-270 of gelonin, while the insert of pING3729 encodes amino acids 184-270 of gelonin plus other 3' sequences.

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(4) Assembly of the overlapping gelonin DNA fragments into a composite gelonin gene

To reassemble the C-terminal two-thirds of the gelonin gene, vector pING3729 was cut with SspI (one SspI site is located within the vector and the second is located about 80 bp downstream of the termination codon of the insert in the vector) and an XhoI linker (8 bp, New England Biolabs) was ligated to the resulting free ends. The DNA was then cut with XhoI and EcoRI, and the 350 bp fragment generated, encoding amino acids 185-270 of gelonin, was isolated. This 350 bp fragment was ligated adjacent to a NcoI to EcoRI fragment from pING3823 encoding amino acids 37-185 of gelonin in a intermediate vector denoted pING3730, thus reassembling the terminal 87% of the gelonin gene (amino acids 37-270).

Next, fragment GEL1-125 was cut with SmaI and NcoI, resulting in a fragment encoding amino acids 1-36 of gelonin which was ligated along with the NcoI to XhoI fragment of pING3730 into the vector pIC100. [pIC100 is identical to pING1500 described in Better et al., *Science*, 240, 1041-1043 (1988), except that it lacks 37 bp upstream of the *pelB* leader sequence. The 37 bp were eliminated by digestion of pING1500 with SphI and EcoRI, treatment with T4 polymerase and religation of the vector. This manipulation regenerated an EcoRI site in the vector while eliminating other undesirable restriction sites.] Before ligation, the vector pIC100 had previously been digested with SstI, treated with T4 polymerase, and cut with XhoI. The ligation generated a new vector containing a complete gelonin gene that was designated plasmid pING3731 (ATCC Accession No. 68721). The complete DNA sequence of the gelonin gene is set out in SEQ ID NO: 11.

Example 2

Construction of Expression Vectors Containing the Gelonin Gene

A first *E. coli* expression vector was constructed containing the gelonin gene linked to the *Erwinia carotovora pelB* leader sequence, and to the *Salmonella typhimurium araB* promoter. A basic vector containing the *araB* promoter is

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described in co-owned U.S. Patent No. 5,028,530 issued July 2, 1991 which is incorporated by reference herein. The vector containing the *araB* promoter was cut with *EcoRI* and *XhoI*. Two DNA fragments were then ligated in tandem immediately downstream of the promoter. The fragment ligated adjacent to the promoter was a 131 bp fragment derived from *SstI* digestion, T4 polymerase treatment and digestion with *EcoRI* of the pIC100 vector which includes the leader sequence of the *E. carotovora pelB* gene. The translated leader sequence is a signal for secretion of the respective protein through the cytoplasmic membrane. The fragment ligated downstream of the leader sequence was a *SmaI* to *XhoI* fragment from pING3731 which contains the complete gelonin gene. Thus, the expression vector contains the gelonin gene linked to the *pelB* leader sequence and the *araB* promoter. This plasmid is designated pING3733.

A second expression vector may be constructed that is identical to the first except that the gelonin gene sequences encoding the nineteen C-terminal amino acids of gelonin are not included. The cDNA sequence of the gelonin gene predicted a 19 residue C-terminal segment that was not detected in any peptide fragments generated for determination of the gelonin amino acid sequence. These 19 amino acids may represent a peptide segment that is cleaved from the mature toxin post-translationally, i.e. that is not present in the native protein. A similar C-terminal amino acid segment was identified in the plant toxin α -trichosanthin [Chow et al., *J. Biol. Chem.*, 265, 8670-8674 (1990)]. Therefore, the expression product without the C-terminal fragment may be of importance.

For construction of a gelonin expression vector without the 19 C-terminal amino acids of gelonin, PCR was used to amplify and alter the 3'-end of the gene. pING3728 was amplified with primers gelo-14 and gelo-9 (SEQ ID NO: 20). The sequence of primer gelo-14 is set out below.

Gelo-14 (SEQ ID NO: 24)

5'TGATCTCGAGTACTATTAGGATCTTTATCGACGA 3'

Primer gelo-14, which corresponds to gelonin amino acids 245-256, introduces a termination codon (underlined in the primer sequence) in the gelonin gene sequence

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which stops transcription of the gene before the sequences encoding the terminal 19 amino acids of the gelonin and also introduces a XhoI site immediately downstream of the termination codon. The PCR product was cut with XhoI and EcoRI, and the resulting 208 bp fragment encoding amino acids 185-251 of gelonin was purified from an agarose gel. This fragment was ligated adjacent to the NcoI to EcoRI fragment from pING3823 encoding amino acids 37-185 of gelonin to generate plasmid pING3732. A final expression vector, pING3734, containing a gelonin gene with an altered 3'-end was generated by substituting an NcoI to XhoI fragment encoding amino acids 37-251 of gelonin from pING3732 into pING3733.

10 Identification of the Native Gelonin 5'-End

Inverse PCR was used to identify a cDNA clone encoding the 5'-end of the mature gelonin gene. 5 µg of total *G. multiflorum* RNA was converted to cDNA using the Superscript Plasmid System (BRL, Gaithersburg, Maryland) with Gelo-11 (SEQ ID NO: 18) as a primer. Gelonin cDNA was self-ligated to generate covalent circular DNA and the ligated DNA was amplified by PCR with oligonucleotides Gelo-9 (SEQ ID NO: 20) and Gelo-16. The sequence of primer Gelo-16 is set out below.

Gelo-16 (SEQ ID NO: 25)

5' GTAAGCAGCATCTGGAGCATCT 3'

20 The PCR product was size-fractionated on an agarose gel and DNAs larger than 300 bp were cloned into SmaI cut pUC18. Several clones were sequenced with the primer Gelo-18, the sequence of which is set out below.

Gelo-18 (SEQ ID NO: 26)

5' CATTCAAGAAATTCACGTAGG 3'

25 A clone identified as having the largest gelonin-specific insert was designated pING3826. The DNA sequence of pING3826 included the first 32 nucleotides of the natural, mature gelonin gene not necessarily present in gelonin expression plasmids pING3733 and pING3734. The complete DNA sequence of the natural gelonin gene is set out in SEQ ID NO: 57.

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Construction of Expression Vectors Containing
a Gelonin Gene with a Natural 5' End

Derivatives of expression vectors pING3733 and pING3734 (described above) containing a gelonin gene with the natural 5' sequence were generated as follows. The 5'-end of gelonin was amplified from pING3826 with the PCR primers Gelo-16 (SEQ ID NO: 24) and Gelo-17, the sequence of which is set out below.

Gelo-17 (SEQ ID NO: 27)

5' GGCCTGGACACCGTGAGCTTTAG 3'

The 285 bp PCR product was treated with T4 polymerase and cut with NcoI. The resulting 100 bp 5'-end DNA fragment was isolated from an agarose gel and ligated adjacent to the 120 bp *pelB* leader fragment from pIC100 (cut with SstI, treated with T4 polymerase and cut with PstI) into either pING3733 or pING3734 digested with PstI and NcoI. The resulting plasmids pING3824 and pING3825 contain the entire native gelonin gene and the native gelonin gene minus the nineteen amino acid carboxyl extension, respectively, linked to the *pelB* leader and under the transcriptional control of the *araB* promoter. The gene construct without the nineteen amino acid carboxyl extension in both pING3734 and pING3825 encodes a protein product referred to in this application as "recombinant gelonin."

Purification of Recombinant Gelonin

Recombinant gelonin was purified by the following procedure: *E. coli* fermentation broth was concentrated and buffer-exchanged to 10 mM sodium phosphate at pH 7.0 by using an S10Y10 cartridge over a DC10 unit (Amicon) the concentrated and buffer-exchanged material was applied to a CM52 column (100 g, 5X10 cm). The column was washed with 1 L of starting buffer and eluted with a 0 to 300 mM NaCl gradient in starting buffer (750 ml total volume). The pure gelonin containing fractions were pooled (elution was from 100-250 mM NaCl), concentrated over an Amicon YM10 membrane, equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and stored frozen at -20°C. A further purification step was attempted using Blue Toyopearl chromatography. However, this procedure did not result in an

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increased purity of material, and resulted in an approximate 50% loss of the starting material.

Example 3

Assembly of gelonin genes with cysteine residues available for conjugation

The gelonin protein has two cysteine residues at positions 44 and 50 which are linked by an endogenous disulfide bond. The protein contains no free cysteine residue directly available for conjugation to antibodies or other proteins. Analogs of gelonin which contain a free cysteine residue available for conjugation were generated by three different approaches. In one approach, various residues along the primary sequence of the gelonin were replaced with a cysteine residue, creating a series of analogs which contain an odd number of cysteine residues. In another approach, one of the two endogenous cysteines was replaced by alanine, creating a molecule which lacks an intrachain disulfide bond but contains a single, unpaired cysteine. In yet another approach both endogenous cysteines were replaced by alanines and a third non-cysteine residue was replaced by a cysteine, creating an analog with a single, unpaired cysteine.

Fifteen analogs of gelonin were constructed. Ten non-cysteine residues of gelonin were targeted for substitution with a cysteine residue. Comparison of the amino acid sequence of gelonin to the natural amino acid sequence and tertiary structure of the ricin A-chain (see FIG. 1) suggested that these positions would be at the surface of the molecule and available for conjugation. Each of the ten gelonin analogs include a cysteine substituted in place of one of the following residues: lysine₁₀, asparagine₄₀, isoleucine₁₀₃, aspartic acid₁₄₆, arginine₁₈₄, serine₂₁₅, asparagine₂₃₉, lysine₂₄₄, aspartic acid₂₄₇, and lysine₂₄₈, and the analogs have respectively been designated Gel_{C10}, Gel_{C40}, Gel_{C103}, Gel_{C146}, Gel_{C184}, Gel_{C215}, Gel_{C239}, Gel_{C244}, Gel_{C247}, and Gel_{C248}.

Two analogs of gelonin were constructed in which one of the native gelonin cysteines that participates in an endogenous disulfide bond was replaced with a non-cysteine residue. Specifically, the cysteine at position 50 was replaced with an

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alanine residue, creating a gelonin analog (designated Gel_{CM}) which has a cysteine available for disulfide bonding at position 44. Conversely, the cysteine at position 44 was replaced with an alanine residue, resulting in an analog (designated Gel_{CS}) which has a cysteine available for disulfide bonding at position 50. The combined series of the foregoing twelve analogs thus spans the entire length of the mature gelonin protein.

Another gelonin analog (Gel_{CMACSM}) was constructed in which both native gelonin cysteines were replaced with alanines. Two additional analogs were constructed that have alanine residues substituted in place of both native cysteines and have either a cysteine residue substituted in place of the native lysine at position 10 (GelC10_{CMACSM}) or a cysteine residue substituted in place of the native aspartate at position 247 (GelC247_{CMACSM}).

The variants of recombinant gelonin were constructed by restriction fragment manipulation or by overlap extension PCR with synthetic oligonucleotides. The sequences of the primers used for PCR are set out below. In each mutagenic primer sequence, the nucleotides corresponding to the changed amino acid, either a cysteine or an alanine residue, are underlined.

Gelo-9 (SEQ ID NO: 20)

Gelo-11 (SEQ ID NO: 18)

Gelo-16 (SEQ ID NO: 25)

Gelo-17 (SEQ ID NO: 27)

Gelo-18 (SEQ ID NO: 26)

Gelo-19 (SEQ ID NO: 60)

5' CAGCCATGGAATCCCATTGCTG 3'

GeloC-1 (SEQ ID NO: 28)

5' TCGATTGCGATCCTAAATAGTACTC 3'

[illegible]

5

5' GTTCGTCTGTAAGATCCTAAATAGTACTCGA 3'

5' GGATCTTTACAGCGAACTTCAAGAGT 3'

5' TCTTGTGCTTCGTCGATAAAGATCC 3'

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5' ATCGACGAAGCAAGAGTGCTATTTT 3'

5' GTAAAACCATGCATAGCACTCTTGAAGTTCGT 3'

5' AGTGCTATGCATGGTTTTACTTGATCAACTGC 3'

15

5' AGCACAIGTGGTGCCACTTATATTACCTA 3'

5' TAAGTGGCACCACATGTGCTAAAGCTCACGGTG 3'

20

5' TGACTGTTGGACAGTTGGCGGAAATA 3'

[illegible]

5

5' GATGATCCTGGAAAGGCTTTCGTTTTGGTAGCGCTT3'

TTTTTTGCGCAGCAATGGG 3'

5' AAGCCTTTCCAGGATCATCAT 3'

5' CACATGTAAAACAAGACTTCATTTTGGC 3'

5' TGAAGTCTTGTTTTAGATGTGTTTTTGAAGAGGCCT3'

5' ATGCCATATGCAATTATAAACCAACGGAGA 3'

5' CATTTCATCAAGTTTCTTG 3'

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5' CTTTCAACAATGCATTGCGCCCGGCGAATAATAC 3'

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GeloC-25 (SEQ ID NO: 66)

5' GCGAATGCAATTGTTGAAAGTTATTTCTAATTTG 3'

GeloC-26 (SEQ ID NO: 67)

5' GTTTTGTGAGGCAGTTGAATTGGAAC 3'

5

GeloC-27 (SEQ ID NO: 68)

5' TTCAACTGCCTCACAACATTCCATTTGCACCT 3'

GeloC-28 (SEQ ID NO: 69)

5' AAAAGCTGATGATCCTGGAAAGTG 3'

10

GeloC-29 (SEQ ID NO: 70)

5' TCCAGGATCATCAGCTTTTTTGCAGCAATGGGA 3'

ara B2 (SEQ ID NO: 43)

5' GCGACTCTCTACTGTTTC 3'

HINDIII-2 (SEQ ID NO: 44)

5' CGTTAGCAATTAACTGTGAT 3'

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(1) Specifically, a cysteine was introduced at amino acid 247 of gelonin (an aspartic acid which corresponds to the cysteine at position 259 in the ricin A-chain) by PCR with mutagenic primers GeloC-3-2 and GeloC-4 in conjunction with primers HINDIII-2 (a primer located in the vector portion of pING3734 or pING3825), Gelo-9 and Gelo-8. Template DNA (pING3734) was amplified with GeloC-3-2 and HINDIII-2 and in a concurrent reaction with GeloC-4 and Gelo-9. The products of these reactions were mixed and amplified with the outside primers Gelo-8 and HINDIII-2. The reaction product was cut with EcoRI and XhoI, purified, and was inserted into plasmid pING3825 in a three-piece ligation. The DNA

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ara B2 and separately with GeloC-20 and HINDIII-2. The products of these reactions were mixed and amplified with the outside primers ara B2 and HINDIII-2. The reaction product was cut with NcoI and BclI, purified, and inserted into pING3825 digested with NcoI and BclI. The oligonucleotides used to place a cysteine at residue 103 also introduced an AflIII restriction site which was verified in the cloned gene. The plasmid containing the Gel_{C103} analog was designated pING3760.

(8) A cysteine was introduced at position 146 (an aspartic acid) by a similar strategy. Template DNA (pING3733) was amplified with mutagenic primer GeloC-22 and Gelo-14 and separately with mutagenic primer GeloC-23 and Gelo-19. The products of these reactions were mixed, and amplified with Gelo-19 and Gelo-14. The reaction product was cut with BglII and EcoRI, and can be inserted into pING3825 in a three-piece ligation. The oligonucleotides used to place a cysteine at residue 146 also introduced a NdeI restriction site which can be verified in the cloned gene.

(9) To introduce a cysteine at position 184 (an arginine) of gelonin, template DNA (pING3733) was amplified with mutagenic primer GeloC-25 and ara B-2 and separately with mutagenic primer GeloC-24 and HINDIII-2. The products of these reactions were mixed, and amplified with ara B2 and Gelo-14. The reaction product was cut with NcoI and BclI, and inserted into pING3825 previously digested with NcoI and BclI. The oligonucleotides used to place a cysteine at residue 184 also introduced an NsiI restriction site which was verified in the cloned gene. The plasmid containing the sequence encoding the Gel_{C184} variant was designated pING3761.

(10) A cysteine can be introduced at position 215 (a serine) by a similar strategy. Template DNA (pING3733) was amplified with mutagenic primer GeloC-27 and ara B2 and separately with mutagenic primer GeloC-26 and HINDIII-2. The products of these reactions were mixed, and amplified with ara B2 and HINDIII-2. The reaction product was cut with EcoRI and BclI, and can be inserted into pING3825 in a three-piece ligation.

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(11) Another gelonin variant with a free cysteine residue was generated by replacing one of the two naturally occurring gelonin cysteine residues, the cysteine at position 50, with an alanine. Plasmid pING3824 was amplified with primers GeloC-17 and Gelo-11, and concurrently in a separate reaction with primers GeloC-19 and ara B2. The reaction products were mixed and amplified with ara B2 and Gelo-11. This product was cut with NcoI and BglII, and cloned back into the vector portion of pING3825 to generate pING3747 (ATCC 69101). This analog was designated Gel_{CM} because it contains a cysteine available for disulfide bonding at amino acid position 44.

(12) A gelonin variant with the natural cysteine at position 44 changed to alanine was constructed by amplifying pING3733 using the mutagenic oligos GeloC-28 and GeloC-29 in conjunction with primers ara B2 and HINDIII-2. The amplified DNA was cut with NcoI and BglII, and cloned into a gelonin vector, generating pING3756. The variant generated was designated Gel_{CSO}.

(13) A gelonin variant in which both the cysteine at position 44 and the cysteine at position 50 of gelonin were changed to alanine residues was constructed by overlap PCR of pING3824 using the mutagenic oligos GeloC-17 and GeloC-18 in conjunction with primers ara B2 and Gelo-11. This analog, like the native gelonin protein, has no cysteine residues available for conjugation. The plasmid encoding the analog was designated pING3750. The analog generated was designated Gel_{CMACS0A}.

(14) The triple mutant GeloninC247_{CMACS0A} was constructed from the plasmids pING3824, pING3750 and pING3737. This variant contains an introduced cysteine at position 247 while both of the naturally occurring cysteine residues at positions 44 and 50 have been replaced with alanine and is desirable because disulfide linkage to an antibody is assured at only a single cysteine residue in the gelonin analog. Plasmid pING3824 was cut with NcoI and XhoI and the vector fragment was purified in an agarose gel. pING3750 was cut with NcoI and EcoRI and pING3737 was cut with EcoRI and XhoI. The NcoI-EcoRI fragment encodes the alanines at positions 44 and 50 while the EcoRI-XhoI fragment encodes the cysteine at position

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247. Each of these fragments was purified and ligated to the NcoI to XhoI vector fragment. The resulting plasmid is named pING3752.

5 (15) The triple mutant GeloninC10_{CMACSA} was also constructed by assembly from previously assembled plasmids. In this case, pING3746 was cut with PstI and NcoI, while pING3750 was cut with NcoI and XhoI. Each of the insert fragments were purified by electrophoresis in an agarose gel, and the fragments were ligated into a PstI and XhoI digested vector fragment. The resulting vector was designated pING3753.

10 Each of the gelonin variants constructed was transformed into *E. coli* strain E104. Upon induction of bacterial cultures with arabinose, gelonin polypeptide could be detected in the culture supernatants with gelonin-specific antibodies. There were no significant differences detected in the expression levels of gelonin from plasmids pING3734 and pING3825, or in the levels from any of the gelonin variants. Each protein was produced in *E. coli* at levels of approximately 1 g/l.

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Example 4

Reticulocyte Lysate Assay

20 The ability of gelonin and recombinant gelonin analogs to inhibit protein synthesis *in vitro* was tested using a reticulocyte lysate assay (RLA) described in Press et al., Immunol. Letters, 14, 37-41 (1986). The assay measures the inhibition of protein synthesis in a cell-free system using endogenous globin mRNA from a rabbit red blood cell lysate. Decreased incorporation of tritiated leucine (³H-Leu) was measured as a function of toxin concentration. Serial log dilutions of standard toxin (the 30 kD form of ricin A-chain, abbreviated as RTA 30), native gelonin, recombinant gelonin (rGelonin) and gelonin analogs were tested over a range of 1 µg/ml to 1 pg/ml. Samples were tested in triplicate, prepared on ice, incubated for 30 minutes at 37°C, and then counted on an Inotec Trace 96 cascade ionization counter. By comparison with an uninhibited sample, the picomolar concentration of toxin (pM) which corresponds to 50% inhibition of protein synthesis (IC₅₀) was calculated. As is shown in Table 2 below, recombinant gelonin and most of its

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analogs exhibit activity in the RLA comparable to that of native gelonin. For some of the analogs (such as Gel_{C239}), RLA activity was diminished.

Table 1

	Toxin	IC ₅₀ (pM)
5	RTA 30	2.5
	Gelonin	15
	rGelonin	11
	Gel _{C10}	60
	Gel _{C44}	20
10	Gel _{C50}	47
	Gel _{C50}	26
	Gel _{C239}	955
	Gel _{C44}	32
	Gel _{C47}	12
15	Gel _{C44}	47
	Gel _{C44C50A}	16
	GelC10 _{C44C50A}	7
	GelC247 _{C44C50A}	20

Example 5**Preparation of Gelonin Immunoconjugates**

Gelonin analogs of the invention were variously conjugated to murine (ATCC HB9286) and chimeric H65 antibody, (CH65) and cH65 antibody domains (including cFab, cFab' and cF(ab')₂ fragments) that are specifically reactive with the human T cell determinant CD5. H65 antibody was prepared and purified by methods described in U.S. Patent Application Serial No. 07/306,433, supra and International Publication No. WO 89/06968, supra. Chimeric H65 antibody was prepared according to methods similar to those described in Robinson et al., Human Antibodies and Hybridomas, 2, 84-93 (1991).

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(1) Conjugation to H65 antibodies

To expose a reactive sulfhydryl, the unpaired cysteine residues of the gelonin analogs were first reduced by incubation with 0.1 to 2 mM DTT (30-60 minutes at room temperature), and then were desalted by size-exclusion chromatography.

Specifically, the Gel_{C248} analog (3.8 mg/ml) was treated with 2 mM DTT for 60 minutes in 0.1 M NaPhosphate, 0.25 M NaCl, pH 7.5 buffer. The Gel_{C244} variant (7.6 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1 M NaPhosphate, 0.25 M NaCl, pH 7.5 buffer. The Gel_{C247} analog (4 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1 M NaPhosphate, 0.5 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. The Gel_{C239} variant (3.2 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1 M NaPhosphate, 0.5 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. The Gel_{C244} analog (4.2 mg/ml) was treated with 0.1 mM DTT for 30 minutes in 0.1 M NaPhosphate, 0.1 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. Lastly, the Gel_{C10} variant (3.1 mg/ml) was treated with 1 mM DTT for 20 minutes in 0.1 M NaPhosphate, 0.1 M NaCl, pH 7.5 buffer with 1 mM EDTA.

The presence of a free sulfhydryl was verified by reaction with DTNB and the average value obtained was 1.4 ± 0.65 SH/molecule. No free thiols were detected in the absence of reduction.

H65 antibody and chimeric H65 antibody were chemically modified with the hindered linker 5-methyl-2-iminothiolane (M2IT) at lysine residues to introduce a reactive sulfhydryl group as described in Goff et al., *Bioconjugate Chem.*, 1, 381-386 (1990).

Specifically, for conjugation with Gel_{C248} and Gel_{C244}, murine H65 antibody at 4 mg/mL was derivitized with 18x M2IT and 2.5 mM DTNB in 25 mM TEOA, 150 mM NaCl, pH 8 buffer for 1 hour at 23°C. The reaction gave 1.9 linkers per antibody as determined by DTNB assay.

For conjugation with Gel_{C247} and Gel_{C239}, H65 antibody at 4.7 mg/mL was derivitized with 20x M2IT and 2.5 mM DTNB in 25 mM TEOA 150 mM NaCl,

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In contrast, when both the recombinant gelonin and the H65 antibody were first derivitized with the crosslinker M2IT (creating gelonin-(M2IT)-SH and H65-(M2IT)-S-S-TNB) and then mixed together, H65-(M2IT)-S-S-(M2IT)-gelonin conjugate was prepared in good yield (toxin/antibody ratio of 1.6). The starting materials for this conjugation (gelonin-(M2IT)-SH and H65-(M2IT)-S-S-TNB) contained linker/protein ratios of 1.2 and 1.4, respectively. Native gelonin was derivitized in a similar manner prior to conjugation to murine or chimeric H65 antibody.

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The reduced gelonin analogs were mixed with H65-(M2IT)-S-S-TNB to allow conjugation. The following conjugation reactions were set up for each analog: 23 mg (in 7.2 ml) of H65-M2IT-TNB were mixed with a 5-fold molar excess of Gel_{C248} (23 mg in 6 ml) for 2 hours at room temperature, then for 18 hours overnight at 4°C; 23 mg (in 7.3 ml) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel_{C244} (23 mg in 3 ml) for 3 hours at room temperature, then for 18 hours overnight at 4°C; 9 mg (in 2.8 mL) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel_{C247} (9 mg in 2.25 mL) for 2 hours at room temperature, then for 5 nights at 4°C; 9 mg (in 2.8 mL) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel_{C239} (9mg in 2.6 mL) for 2 hours at room temperature, then at 4°C for 3 days; 12 mg (in 1.9 mL) of H65-m2IT-TNB were mixed with a 5.6-fold molar excess of Gel_{C24} (13.44 mg in 3.2 mL) for 4.5 hours at room temperature, then 4°C overnight; and 11 mg of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel_{C10} (11 mg in 3.5 mL) for 4 hours at room temperature, then at 4°C overnight.

Following conjugation, unreacted M2IT linkers on the antibody were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was then loaded onto a gel filtration column [Sephadex G-150 (Pharmacia) in the case of Gel_{C248}, Gel_{C247}, Gel_{C244} and Gel_{C239} and an Aca-44 column (IBF Biotechnics, France) in the case of Gel_{C24} and Gel_{C10}]. The reactions were run over the gel filtration columns and eluted with 10 mM Tris, 0.15M NaCl pH 7. The first peak off each column was loaded onto Blue Toyopearl® resin (TosoHaas, Philadelphia, Pennsylvania) in 10 mM Tris, 30 mM NaCl, pH 7 and the product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

Samples of the final conjugation products were run on 5% non-reduced SDS PAGE, Coomassie stained and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody (T/A ratio). The yield of final product for each analog conjugate was as follows: Gel_{C248}, 17 mg with a T/A ratio of 1.6; Gel_{C247}, 1.1 mg with a T/A ratio of 1; Gel_{C244}, 4.5 mgs with a T/A ratio of 1.46; Gel_{C239}, 2.9 mg with a T/A ratio of 2.4; Gel_{C24}, 7.3 mg with a T/A ratio of 1.22; and Gel_{C10}, 6.2 mg with a T/A ratio of 1.37. Conjugation efficiency (i.e., conversion of

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free antibody to immunoconjugate) was significantly greater (~ 80%) for some analogs (Gel_{C10}, Gel_{C4}, Gel_{C29}, Gel_{C27}, and Gel_{C44}) than for others (~ 10%, Gel_{C44}).

(2) Conjugation to antibody fragments

5 Analog Gel_{C27} and Gel_{C4} were conjugated to various chimeric [cFab, cFab' and cF(ab')₂] and "human engineered" [he1 Fab, he2 Fab, he3 Fab, he1 Fab' and he1 F(ab')₂] antibody fragments. Chimeric H65 antibody fragments may be prepared according to the methods described in U.S. Patent Application Serial No. 07/714,175, *supra* and in International Publication No. WO 89/00999, *supra*. The DNA sequences encoding the variable regions of H65 antibody fragments that were
10 human engineered (referring to the replacement of selected murine-encoded amino acids to make the H65 antibody sequences less immunogenic to humans) according to the methods described in co-pending, co-owned U.S. Patent Application Serial No. 07/808,454 filed December 13, 1991 which is incorporated by reference herein, are
15 set out in SEQ ID NO: 71 (the kappa chain of he1 and he2), SEQ ID NO: 72 (the gamma chain of he1), SEQ ID NO: 73 (the gamma chain of he2 and he3) and SEQ ID NO: 74 (the kappa chain of he3)

The chimeric H65 antibody fragments were conjugated to Gel_{C27} analog basically as described below for conjugation of human engineered Fab and Fab' fragments to Gel_{C27} and Gel_{C4}.

20 (a) he1 Fab-Gel_{C27}

The he1 Fab was dialyzed into 25 mM TEOA buffer, 250 mM NaCl, pH 8 and then concentrated to 6.8 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used at 20-fold molar excess, in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 30 minutes at
25 room temperature, then desalted on GF05 (gel filtration resin) and equilibrated in 0.1 M Na Phosphate, 0.2M NaCl, pH 7.5. A linker number of 1.8 linkers per Fab was calculated based on the DTNB assay. The he1 Fab-M2IT-TNB was concentrated to 3.7 mg/mL prior to conjugation with Gel_{C27}.

30 Gel_{C27} at 12.8 mg/mL in 10 mM Na Phosphate, 0.3M NaCl, was treated with 1 mM DTT, 0.5 mM EDTA for 20 minutes at room temperature to

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expose a reactive sulfhydryl for conjugation and then was desalted on GF05 and equilibrated in 0.1 M Na Phosphate, 0.2 M NaCl, pH 7.5. Free thiol content was determined to be 0.74 moles of free SH per mole of Gel_{C247} using the DTNB assay. The gelonin was concentrated to 8.3 mg/mL prior to conjugation with activated antibody.

The conjugation reaction between the free thiol on Gel_{C247} and the derivitized hel Fab-M2IT-TNB, conditions were as follows. A 5-fold excess of the gelonin analog was added to activated hel Fab-M2IT-TNB (both proteins were in 0.1M Na Phosphate, 0.2M NaCl, pH7.5) and the reaction mixture was incubated for 3.5 hours at room temperature and then overnight at 4°C. Following conjugation, untreated M2IT linkers were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was loaded onto a gel filtration column (G-75) equilibrated with 10 mM Tris, 150 mM NaCl, pH 7. The first peak off this column was diluted to 30 mM NaCl with 10 mM Tris, pH7 and loaded on Blue Toyopearl®. The product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

(b) hel Fab'-Gel_{C247}

Similarly, the H65 hel Fab' fragment was dialyzed into 25 mM TEOA buffer, 400 mM NaCl, pH 8 at 2.9 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used at 20-fold molar excess, in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 1 hour at room temperature then it was desalted on GF05 (gel filtration resin) and equilibrated in 0.1 M Na Phosphate, 0.2 M NaCl, pH 7.5. A linker number of 1.6 linkers per Fab' was calculated based on the DTNB assay. The hel Fab'-M2IT-TNB was concentrated to 3.7 mg/mL prior to conjugation with Gel_{C247}.

The Gel_{C247} at 77 mg/mL was diluted with in 10 mM Na Phosphate, 0.1 M NaCl to a concentration of 5 mg/mL, treated with 1 mM DTT, 0.5 mM EDTA for 30 minutes at room temperature to expose a free thiol for conjugation and then was desalted on GF05 and equilibrated in 0.1 M Na Phosphate, 0.2 M NaCl, pH 7.5. Free thiol content was determined to be 1.48 moles of free SH per mole of

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Gel_{CM} using the DTNB assay. The gelonin was concentrated to 11.4 mg/mL prior to conjugation with activated he2 Fab.

5 The conjugation reaction conditions between the free thiol on Gel_{CM} and the derivitized he2 Fab-M2IT-TNB were as follows. A 3-fold excess of the gelonin analog was added to activated he2 Fab-M2IT-TNB (both proteins were in 0.1 M Na Phosphate, 0.1 M NaCl, pH 7.5 but the gelonin solution contained 0.5 mM EDTA as well). The reaction mixture was concentrated to half its original volume, then the mixture was incubated for 4 hours at room temperature followed by 72 hours at 4°C. Following the incubation period the efficiency of conjugation was estimated
10 at 70-75% by examination of SDS PAGE.

Following conjugation the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction as loaded onto a gel filtration column (G-75) equilibrated in 10 mM Tris, 0.15 M NaCl, pH 7. The first peak off this column was diluted to 30
15 mM NaCl with 10 mM Tris, pH 7 and loaded onto a Blue Toyopearl® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

(d) he3 Fab Gel_{CM}

Similarly, the he3 Fab was dialyzed overnight into 25 mM TEOA, 0.25 M NaCl, pH 8 buffer and then concentrated to 5 mg/mL prior to derivitization with
20 the M2IT crosslinker. For the linker reaction, M2IT was used in a 10-fold molar excess in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 45 minutes at room temperature and was then desalted on a GF05-LS (gel filtration) column, equilibrated in 0.1 M Na Phosphate, 0.2 M NaCl with 0.02% Na azide. A linker number of 1 M2IT per Fab-M2IT-TNB was calculated based on the DTNB
25 assay. After derivitization and gel filtration, the he3 Fab concentration was 5.3 mg/mL.

Gel_{CM} at 7.8 mg/mL in 0.1 M Na Phosphate, 0.1 M NaCl, pH 7.5 was treated with 1.5 mM DTT and 1 mM EDTA for 30 minutes at room temperature to expose a reactive thiol for conjugation and then was desalted on GF05-LS resin
30 equilibrated in 0.1 M Na Phosphate, 0.1 M NaCl plus 0.02% Na azide, pH 7.5.

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Free thiol content was determined to be 0.66 moles of free SH per mole of Gel_{CM} using the DTNB assay. The gelonin was concentrated to 5.2 mg/mL prior to conjugation with activated he3 Fab.

5 The conjugation reaction conditions between the free thiol on Gel_{CM} and the derivitized he3 Fab-M2IT-TNB were as follows. A 5-fold excess of the gelonin analog was added to activated he3 Fab-M2IT-TNB (both proteins were in 0.1 M Na phosphate 0.1 M NaCl, pH 7.5). The reaction mixture was incubated for 2 hours at room temperature followed by 72 hour at 4°C. Following the incubated period the efficiency of conjugation was estimated at 70-75% by examination of SDS
10 PAGE.

Following conjugation, the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction was loaded onto a GammaBind G (immobilized protein G affinity resin, obtained from Genex, Gaithersburg, Maryland) equilibrated in 10 mM
15 Na Phosphate, 0.15 M NaCl, pH 7. It was eluted with 0.5 M NaOAc, pH 3 and neutralized with Tris. It was dialyzed into 10 mM Tris, 0.15 M NaCl, pH 7 overnight, then diluted to 30 mM NaCl with 10 mM Tris, pH 7 and loaded onto a blue Toyopearl® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5

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Example 6

Whole Cell Kill Assays

Immunoconjugates prepared with gelonin and gelonin analogs were tested for cytotoxicity against an acute lymphoblastoid leukemia T cell line (HSB2 cells) and against human peripheral blood mononuclear cells (PBMCs).
25 Immunoconjugates of ricin A-chain with H65 antibody (H65-RTA) and antibody fragments were also tested. The ricin A-chain (RTA) as well as the H65-RTA immunoconjugates were prepared and purified according to methods described in U.S. Patent Application Serial No. 07/306,433, supra and in International Publication No. WO 89/06968, supra.

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Briefly, HSB2 cells were incubated with immunotoxin and the inhibition of protein synthesis in the presence of immunotoxin was measured relative to untreated control cells. The standard immunoconjugates H65-RTA (H65 derivitized with SPDP linked to RTA), H65-Gelonin and H65-rGelonin, H65 fragment immunoconjugate, and gelonin immunoconjugate samples were diluted with RPMI without leucine at half-log concentrations ranging from 2000 to 0.632 ng/ml. All dilutions were added in triplicate to microtiter plates containing 1×10^5 HSB2 cells. HSB2 plates were incubated for 20 hours at 37°C and then pulsed with ^3H -Leu for 4 hours before harvesting. Samples were counted on the Inotec Trace 96 cascade ionization counter. By comparison with an untreated sample, the picomolar concentration (pM) of immunotoxin which resulted in a 50% inhibition of protein synthesis (IC_{50}) was calculated. In order to normalize for conjugates containing differing amounts of toxin or toxin analog, the cytotoxicity data were converted to picomolar toxin (pM T) by multiplying the conjugate IC_{50} (in pM) by the toxin/antibody ratio which is unique to each conjugate preparation.

The PMBC assays were performed as described by Fishwild et al., Clin. and Exp. Immunol., 86, 506-513 (1991) and involved the incubation of immunoconjugates with PBMCs for a total of 90 hours. During the final 16 hours of incubation, ^3H -thymidine was added; upon completion, immunoconjugate-induced inhibition of DNA synthesis was quantified. The activities of the H65 and chimeric H65 antibody conjugates against HSB2 cells and PBMC cells are listed in Table 2 below.

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Table 2
IC₅₀ (pM T)

	Conjugate	HSB2 Cells	PBMCs
	H65-RTA	143	459
5	H65-(M2IT)-S-S-(M2IT)-Gelolin	1770	81
	H65-(M2IT)-S-S-(M2IT)-rGelolin	276	75
	H65-(M2IT)-S-S-Gel _{C10}	140	28
	H65-(M2IT)-S-S-Gel _{C44}	99	51
	H65-(M2IT)-S-S-Gel _{C39}	2328	180
10	H65-(M2IT)-S-S-Gel _{C44}	> 5000	> 2700
	H65-(M2IT)-S-S-Gel _{C47}	41	35
	H65-(M2IT)-S-S-Gel _{C48}	440	203
	cH65-RTA ₃₀	60	400
	cH65-(M2IT)-S-S-(M2IT)-Gelolin	1770	140
15	cH65-(M2IT)-S-S-(M2IT)-rGelolin	153	120
	cH65-(M2IT)-S-S-Gel _{C39}	> 7000	290
	cH65-(M2IT)-S-S-Gel _{C47}	34	60
	cH65-(M2IT)-S-S-Gel _{C48}	238	860

20 Against HSB2 cells, many of the gelonin analog immunoconjugates were significantly more potent than conjugates prepared with native gelonin or recombinant, unmodified gelonin, both in terms of a low IC₅₀ value, but also in terms of a greater extent of cell kill. Against human PBMCs, the gelonin analog conjugates were at least as active as native and recombinant gelonin conjugates. Importantly, however, some of the conjugates (for example, Gel_{C10}, Gel_{C44} and Gel_{C47}) exhibited

25 an enhanced potency against PBMCs compared to native and recombinant gelonin conjugates, and also exhibited an enhanced level of cell kill (data not shown).

The activities of the H65 antibody fragment conjugates against HSB2 cells and PBMC cells are listed in Tables 3 and 4 below, wherein extent of kill in

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Table 4 refers to the percentage of protein synthesis inhibited in HSB2 cells at the highest immunotoxin concentration tested (1 $\mu\text{g/ml}$).

Table 3

IC₅₀ (pM T)

5	Conjugate	HSB2 Cells	PBMCs
	cFab'-RTA 30	530	1800
	cFab'-rGelolin	135	160
	cFab'-Gel _{C247}	48	64
	cF(ab') ₂ -RTA 30	33	57
10	cF(ab') ₂ -rGelolin	55	34
	cF(ab') ₂ -Gel _{C247}	23	20
	cF(ab') ₂ -Gel _{C44}	181	95

Table 4

IC₅₀ (pM T)

15	Conjugate	HSB2 Cells	Extent of Kill
	he1 Fab'-Gel _{C247}	57.7	93%
	he1 Fab-Gel _{C247}	180	94%
	he2 Fab-Gel _{C44}	363	91%
	he3 Fab-Gel _{C44}	191	93%
20	cFab'-Gel _{C247}	47.5	93%
	cF(ab') ₂ -rGelolin	45.4	85%
	F(ab') ₂ -Gel _{C247}	77.5	83%
	cF(ab') ₂ -Gel _{C247}	23.2	85%

The cFab'-Gel_{C247} immunoconjugate is clearly more cytotoxic than cFab' conjugates with recombinant gelonin or RTA 30.

Recombinant gelonin and the gelonin analogs exhibited enhanced solubility in comparison to both native gelonin and RTA30. In addition, recombinant gelonin and gelonin analog immunoconjugates exhibited enhanced solubility relative to immunoconjugates prepared with native gelonin and RTA30. This enhanced solubility was particularly noteworthy for recombinant gelonin and analog conjugates prepared with chimeric Fab fragments.

10 The stability of the disulfide bond linking a RIP to a targeting molecule (such as an antibody) is known to influence the lifespan of immunoconjugates in vivo [see Thorpe et al., Cancer Res., 47, 5924-5931 (1987)]. For example, conjugates in which the disulfide bond is easily broken by reduction in vitro are less stable and less efficacious in animal models [see Thorpe et al., Cancer Res., 48, 6396-6403 (1988)].

By comparison with the amount of RIP released by high concentrations of 2-mercaptoethanol (to determine 100% release), the concentration of glutathione required to release 50% of the RIP (the RC_{50}) was calculated. The results of assays for H65 antibody conjugates are set out in Table 5 below.

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Table 5

	Conjugate	RC ₅₀ (mM)
	H65-RTA 30	3.2
	H65-(M2IT)-S-S-(M2IT)-gelonin	11.1
5	H65-(M2IT)-S-S-(M2IT)-rGelonin	3.0
	H65-(M2IT)-S-S-Gel _{C10}	2.5
	H65-(M2IT)-S-S-Gel _{C44}	0.6
	H65-(M2IT)-S-S-Gel _{C239}	774.0
	H65-(M2IT)-S-S-Gel _{C44}	1.2
10	H65-(M2IT)-S-S-Gel _{C47}	0.1
	H65-(M2IT)-S-S-Gel _{C44}	0.4
	cH65-RTA 30	2.50
	cH65-(M2IT)-S-S-(M2IT)-rGelonin	2.39
	cH65-(M2IT)-S-S-Gel _{C47}	0.11
15	cH65-(M2IT)-S-S-Gel _{C44}	0.32

The foregoing results indicate that the stability of the bonds between the different gelonin proteins and H65 antibody varied greatly. With the exception of Gel_{C10} and Gel_{C239}, most of the gelonin analogs resulted in conjugates with linkages that were somewhat less stable in this in vitro assay than the dual-linker chemical conjugate.

20 The stability of the Gel_{C239} analog, however, was particularly enhanced.

The results of the assay for H65 antibody fragment conjugates are set out in Table 6 below.

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Table 6

	<u>Conjugate</u>	<u>RC₅₀ (mM)</u>
	he1 Fab'-Gel _{C247}	0.07
	cFab'-Gel _{onin}	1.27
5	cFab'-Gel _{C247}	0.08
	cF(ab') ₂ -RTA 30	1.74
	cF(ab') ₂ -rGel _{onin}	2.30
	cF(ab') ₂ -Gel _{C247}	0.09
	cF(ab') ₂ -Gel _{C248}	0.32
10	he2 Fab-Gel _{C44}	0.46
	he3 Fab-Gel _{C44}	0.58

From the RC₅₀ results presented in Tables 5 and 6, it appears that the particular RIP analog component of each immunotoxin dictates the stability of the immunotoxin disulfide bond in vitro.

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Example 8**Pharmacokinetics of Conjugates to H65 Antibody**

The pharmacokinetics of gelonin analogs Gel_{C247}, Gel_{C44} and Gel_{C10} linked to whole H65 antibody was investigated in rats. An IV bolus of 0.1 mg/kg of ¹²⁵I-labelled immunoconjugate H65-(M2IT)-S-S-Gel_{C247}, H65-(M2IT)-S-S-Gel_{C44} or H65-(M2IT)-S-S-Gel_{C10} was administered to male Sprague-Dawley rats weighing 134-148 grams. Serum samples were collected from the rats at 3, 15, 30 and 45 minutes, and at 1.5, 2, 4, 6, 8, 18, 24, 48, 72, and 96 hours. Radioactivity (cpm/ml) of each sample was measured, and SDS-PAGE was performed to determine the fraction of radioactivity associated with whole immunoconjugate. Immunoconjugate associated serum radioactivity was analyzed using the computer program PCNONLIN (SCI Software, Lexington, Kentucky). Table 7 below lists the pharmacokinetic parameters of the immunoconjugates. In the table, the standard error for each value is indicated and a one way analysis of variance is presented, IC is the immunoconjugate (specified

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by the abbreviation for the gelonin variant that is part of the immunoconjugate) n is the number of animals in the study, Vc is the central volume of distribution, Cl is the clearance, MRT is the total body mean residence time, Alpha is the α half-life and Beta is the β half-life of the immunoconjugate.

5

Table 7

IC	Vc (ml/kg)	Cl (ml/hr/kg)	MRT (hours)	Alpha (hours)	Beta (hours)
H65 Gel _{C247} n=32	65.3 \pm 3.4	11.0 \pm 0.4	16.5 \pm 1.9	2.3 \pm 0.2	20.5 \pm 3.0
10 H65 Gel _{C44} n=38	61.9 \pm 2.4	4.1 \pm 0.1	22.7 \pm 0.7	3.0 \pm 0.7	17.8 \pm 0.8
H65 Gel _{C10} n=45	59.2 \pm 1.3	2.5 \pm 0.04	42.7 \pm 1.1	3.3 \pm 0.3	32.9 \pm 1.1
p-value	0.176	<0.0001	<0.0001	0.303	<0.0001

15

The Gel_{C247} immunoconjugate was found to have α and β half lives of 2.3 and 20 hours, with a total mean residence time of 17 hours. The 72 and 96 hour time points were excluded from analysis because of the poor resolution of immunoconjugate associated radioactivity on the SDS-PAGE gel for these serum samples.

20

Because in vitro studies suggested that the Gel_{C10} immunoconjugate had greater disulfide bond stability, it was anticipated that its half lives in vivo would be longer relative to the cys₂₄₇ form of the immunoconjugate. The β half life of the immunoconjugate was about 33 hours compared to 20 hours for the Gel_{C247} conjugate. The total mean residence time was also much greater for the Gel_{C10} immunoconjugate (42 hours versus 42 hours for the Gel_{C247} conjugate). In addition, the clearance of the Gel_{C10} immunoconjugate was 2.5 ml/hr/kg, about four times less than that of the Gel_{C247} immunoconjugate (11 ml/hr/kg). As also predicted from the in vitro disulfide

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stability data, the clearance of the Gel_{CM} immunoconjugate was intermediate between those of the Gel_{C10} and Gel_{C27} immunoconjugates.

Based on these studies, the Gel_{C10} analog conjugated to H65 antibody has greater *in vivo* stability than the Gel_{CM} and Gel_{C27} analogs conjugated to H65 antibody (as determined by the longer mean residence time and clearance rates), although the properties of the Gel_{CM} immunoconjugate more closely resembled those of the Gel_{C10} immunoconjugate than the Gel_{C27} immunoconjugate.

Pharmacokinetics of Conjugates to H65 Antibody Fragments

The pharmacokinetics of Gel_{C27} and Gel_{CM} analogs linked to human engineered H65 Fab fragments were also investigated in rats. An IV bolus of 0.1 mg/kg of ¹²⁵I-labelled he1 H65 Fab-Gel_{C27}, he2 H65 Fab-Gel_{CM} or he3 H65 Fab-Gel_{CM} was administered to male Sprague-Dawley rats weighing 150-180 grams. Serum samples were collected at 3, 5, 15, 20, 30, and 40 minutes, and 1, 1.5, 3, 6, 8, 18, 24, 32, 48, and 72 hours, and were analyzed by ELISA using rabbit anti-Gelonin antibody as the capture antibody and biotin-labelled goat anti-human kappa light chain antibody as the secondary antibody. Results of the analysis are presented in Table 8 below. In the table, the standard error for each value is shown, and IC is the immunoconjugate, n is the number of animals in the study, Vc is the central volume of distribution, Vss is the steady state volume of distribution, Cl is the clearance, MRT is the total body mean residence time, Alpha is the α half-life and Beta is the β half-life of the indicated conjugate.

Table 8

IC	Vc (ml/kg)	Vss (ml/hr/kg)	Cl (ml/hr/kg)	MRT (hours)	Alpha (hours)	Beta (hours)
25 he1 Gel _{C27} n=27	48 ± 3	133 ± 7	62 ± 3	2.1 ± 0.1	0.33 ± 0.03	3.0 fixed
he2 Gel _{CM} n=28	54 ± 5	141 ± 8	53 ± 3	2.7 ± 0.2	0.37 ± 0.04	3.1 fixed
30 he3 Gel _{CM} n=33	77 ± 6	140 ± 20	57 ± 3	2.5 ± 0.4	0.58 ± 0.11	3.0 ± 1.0

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Comparing the three immunoconjugates, the pharmacokinetics of he1 H65 Fab-Gel_{CM7}, he2 H65 Fab-Gel_{CM4} and he3 Fab-Gel_{CM4} were very similar, having similar alpha and beta half-lives, mean residence times, and clearance, particularly when comparing parameters obtained from the ELISA assayed curves. This is in contrast to their whole antibody immunoconjugate counterparts, where the clearance of Gel_{CM7} immunoconjugate (11 ml/kg/hr) was three-fold greater than that of Gel_{CM4} immunoconjugate (4 ml/kg/hr). This suggests that cleavage of the disulfide bond linking the Fab fragment and gelonin is not as important for the serum clearance of Fab immunoconjugates as for whole antibody immunoconjugates.

Immunogenicity of Immunoconjugates

Outbred Swiss/Webster mice were injected repeatedly (0.2 mg/kg each injection) with murine H65 antibody conjugates prepared with RTA, RTA30 and recombinant gelonin. The cycle was such that each animal was injected on days 1 and 2, and then the injections were repeated 28 and 29 days later. The animals received 5 such cycles of injections. One week and three weeks following each series of injections, blood was collected and the amount of anti-RIP antibodies present was determined by ELISA; peak titers for each cycle are shown in Table 9. RTA and RTA30 generated strong responses which began immediately following the first cycle of injections and remained high throughout the experiment. In contrast, no immune response was detected for the gelonin conjugate, even after 5 cycles of injections. When the conjugates were mixed with Complete Freund Adjuvant and injected i.p. into mice, anti-RTA and RTA-30 antibodies were readily detected after several weeks. These data indicate that anti-gelonin antibodies, if generated, would have been detected by the ELISA assay, and suggest that recombinant gelonin may be much less immunogenic in animals than is RTA.

	<u>Cycle</u>	<u>H65-RTA</u>	<u>H65-RTA30</u>	<u>H65-rGel</u>
	Prebleed	100	100	100
	Cycle 1	168	117	100
5	Cycle 2	4208	1008	100
	Cycle 3	7468	3586	100
	Cycle 4	5707	3936	100
	Cycle 5	4042	2505	100

10 A human peripheral blood lymphocyte (PBL)-reconstituted, severe combined immunodeficient mouse model was utilized to evaluate the *in vivo* efficacy of various immunoconjugates comprising the gelonin analogs Gel_{CM7} and Gel_{CM}. Immunoconjugates were tested for the capacity to deplete human blood cells expressing the CD5 antigen.

Human peripheral blood cells were obtained from lymphapheresis samples (HemaCare Corporation, Sherman Oaks, CA) or venous blood samples (Stanford University Blood Bank, Palo Alto, CA) collected from healthy donors. Blood cells were enriched for PBLs using Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque®; Pharmacia, Piscataway, New Jersey) and subsequently washed 4 times with PBS. Residual erythrocytes were lysed with RBC lysing buffer (16 μ M ammonium chloride, 1 mM potassium bicarbonate, 12.5 μ M EDTA) during the second wash. Cell viability in the final suspension was >95% as assessed by trypan blue dye exclusion.

CB.17 scid/scid (SCID) mice were purchased from Taconic (Germantown, New York) or were bred under sterile conditions in a specific pathogen-free animal facility (original breeding pairs were obtained from Hana

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Biologics, Alameda, California). Animals were housed in filter-top cages and were not administered prophylactic antibiotic treatment. Cages, bedding, food and water were autoclaved before use. All manipulations with animals were performed in a laminar flow hood.

- 5 Untreated SCID mice were bled for determination of mouse Ig levels. Human PBL-injected mice were bled at various intervals for quantitation of human Ig and sIL-2R. Blood collection was from the retro-orbital sinus into heparinized tubes. Blood samples were centrifuged at 300 x g for 10 min, and plasma was collected and stored at -70°C. Mouse and human Ig were quantified using standard
- 10 sandwich ELISAs. Briefly, flat-bottom microtiter plates (MaxiSorp Immuno-Plates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with goat anti-mouse IgG+IgA+IgM (Zymed Laboratories, Inc., South San Francisco, California) or goat anti-human Igs (Tago, Inc., Burlingame, California) in bicarbonate buffer, pH 9.6. Plates were blocked for 2 hours at room temperature with 1% BSA in Tris-buffered
- 15 saline, pH 7.5 (TBS), and then incubated at 37°C for 1 hour with standards or samples serially-diluted in TBS/1% BSA/0.05% Tween 20. Standards used were a monoclonal mouse IgG2a (IND1 anti-melanoma; XOMA Corporation, Berkeley, California) and polyclonal human Ig (Sigma Chemical Co., St. Louis, Missouri). Subsequently, plates were washed with TBS/Tween 20 and incubated at 37°C for 1
- 20 hour with alkaline phosphatase-conjugated goat anti-mouse IgG+IgA+IgM or goat anti-human Igs (Caltag Laboratories, South San Francisco, California). Detection was by measurement of absorbance at 405 nm following incubation with 1 mg/ml p-nitrophenylphosphate (Sigma) in 10% diethanolamine buffer, pH 9.8. Plasma from a normal BALB/c mouse was used as a positive control in the mouse Ig ELISA.
- 25 Plasma samples from naive SCID mice or normal BALB/c mice did not have detectable levels of human Ig. Human sIL-2R was quantified using an ELISA kit (Immunotech S.A., Marseille, France) as per the manufacturer's instructions.

Five-to-seven week old mice with low plasma levels of mouse Ig (<10µg/ml) were preconditioned with an i.p. injection of cyclophosphamide (Sigma)

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at 200 mg/kg. Two days later, they were injected i.p. with $25-40 \times 10^6$ freshly-isolated human PBL suspended in 0.8 ml PBS.

Immunoconjugate Treatment

SCID mice were bled at approximately 2 weeks after human PBL transplantation. Mice with undetectable < 10 pM or low plasma levels of human sIL-2R were eliminated from the study. The cut-off for exclusion of mice with detectable, but low, levels of human sIL-2R was empirically determined for each study and was generally 20 pM. The remaining mice were divided into groups and were administered vehicle or immunoconjugate as an i.v. bolus (0.2 mg/kg) daily for 5 consecutive days. Animals were sacrificed 1 day after cessation of treatment for quantitation of human T cells in tissues and human sIL-2R in plasma.

Collection of Tissues and Analysis of PBL Depletion

Blood was collected from the retro-orbital sinus into heparinized tubes. Mice were then killed by cervical dislocation and spleens were removed aseptically. Single cell suspensions of splenocytes were prepared in HBSS by pressing the spleens between the frosted ends of sterile glass microscope slides. Collected cells were washed twice with PBS. Erythrocytes were eliminated from blood and splenocyte suspensions using RBC lysing buffer. Subsequently, cells were resuspended in PBS for enumeration. Recovered cells were then assayed for Ag expression using flow cytometry.

Two to five hundred thousand cells in 100 μ l of PBS/1% BSA/0.1% sodium azide were incubated on ice for 30 min. with saturating amounts of various FITC- or phycoerythrin (PE)-conjugated Abs (Becton-Dickinson, Mountain View, CA). Abs used for staining included: HLe-1-FITC (IgG1 anti-CD45), Leu 2-FITC (IgG1 anti-CD8), Leu 3 PE (IgG1 anti-CD4), and Leu M3-PE (IgG2a anti-CD14). Cells were then washed in cold buffer and fixed in 0.37% formaldehyde in PBS. Samples were analyzed on a FACscan (Becton-Dickinson) using log amplifiers. Regions to quantify positive cells were set based on staining of cells obtained from naive SCID mice. The absolute numbers of human Ag-positive cells recovered from SCID tissues were determined by multiplying the percent positive cells by the total

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number of cells recovered from each tissue sample. The total number of leukocytes in blood was calculated using a theoretical blood volume of 1.4 ml/mouse. The detection limit for accurate quantitation of human cells in SCID mouse tissues was 0.05%. All statistical comparison between treatment groups were made using the Mann-Whitney U test. Treatment groups were determined to be significantly different from buffer control groups when the p value was <0.05. Results are presented in Table 10 below, wherein + indicates a significant difference from controls, - indicates an insignificant difference and NT means the conjugate was not tested. CD5 Plus (XOMA Corporation, Berkeley, California) is mouse H65 antibody chemically linked to RTA and is a positive control. OX19 Fab-Gel₂₄₇ is a negative control immunoconjugate. The OX19 antibody (European Collection of Animal Cell Cultures #84112012) is a mouse anti-rat CD5 antibody that does not cross react with human CD5.

Table 10

15	Test Article	Human T Cell Depletion	
		<u>Spleen</u>	<u>Blood</u>
	CD5 Plus	+	+
	cH65 F(ab') ₂	-	-
	cH65 Fab'	-	-
20	H65-rGEL	+	+
	cH65 F(ab') ₂ -rGel	+	+
	cH65 Fab'-rGel	+	+
	cH65 F(ab') ₂ -Gel ₂₄₇	+	NT
	cH65 Fab'-Gel ₂₄₇	+	+
25	he1H65 Fab'-Gel ₂₄₇	+	NT
	cH65 Fab'-Gel ₂₄₄	+	+
	OX19 Fab-Gel ₂₄₇	-	-

All the gelonin immunoconjugates were capable of depleting human cells in the mouse model.

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Example 10

Nine genetic constructs were assembled that each included a natural sequence gelonin gene fused to an H65 truncated heavy chain gene (Fd) or an H65 light chain gene (kappa). The H65 Fd sequence consists of the nucleotides encoding the murine H65 heavy chain variable (V), joining (J) and human IgG₁, constant (C) domain 1 regions. The DNA sequences of the V and J regions of the H65 Fd and kappa fragment genes linked to the pel B leader can be obtained from GenBank (Los Alamos National Laboratories, Los Alamos, New Mexico) under Accession Nos. M90468 and M90467, respectively. Four of the gene fusions included a gelonin gene linked at the 5' end of an H65 Fab fragment gene while the other four included an gelonin gene linked at the 3' end of an H65 Fab fragment gene. A DNA linker encoding a peptide segment of the E. coli shiga-like toxin (SLT) (SEQ ID NO: 58), which contains two cysteine residues participating in a disulfide bond and forming a loop that includes a protease sensitive amino acid sequence) or of rabbit muscle aldolase (RMA) (SEQ ID NO: 59), which contains several potential cathepsin cleavage sites) was inserted between the gelonin gene and the antibody gene in the constructs. Alternatively, a direct fusion was made between a gelonin gene and an H65 Fab fragment gene without a peptide linker segment. Table 11 below sets out a descriptive name of each gene fusion and indicates the expression plasmid containing the gene fusion. Each plasmid also includes the Fab fragment gene (shown in parentheses in Table 11) with which each particular gene fusion was co-expressed.

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Table 11

	<u>Plasmid</u>	<u>Description</u>
	pING3754	Gelonin::SLT::Fd (kappa)
	pING3757	Gelonin::SLT::kappa (Fd)
5	pING3759	Gelonin::RMA::Fd (kappa)
	pING3758	Gelonin::RMA::kappa (Fd)
	pING4406	Fd::SLT::Gelonin (kappa)
	pING4407	kappa::SLT::Gelonin (Fd)
	pING4408	Fd::RMA::Gelonin (kappa)
10	pING4410	kappa::RMA::Gelonin (Fd)
	pING3334	Gelonin::Fd (kappa)

Fusions of Gelonin at the Carboxyl-Terminus of Antibody Genes

(1) Fd::SLT::Gelonin (kappa)

15 A gelonin gene fusion to the 3'-end of the H65 Fd chain with the 23 amino acid SLT linker sequence was assembled in a three piece ligation from plasmids pVK1, pING3731 (ATCC 68721) and pING4000. Plasmid pVK1 contains the Fd gene linked in-frame to the SLT linker sequence; pING3731 contains the gelonin gene, and pING4000 contains the H65 kappa and Fd genes each linked to the pelB leader sequence under the control of the araB promoter as a dicistronic message.

20 Plasmid pVK1 was designed to link the 3'-end of a human IgG Fd₁ constant region in-frame to a protease-sensitive segment of the SLT gene bounded by two cysteine residues which form an intra-chain disulfide bond. The SLT gene segment (20 amino acids from SLT bounded by cysteine residues, plus three amino acids introduced to facilitate cloning) was assembled from two oligonucleotides, SLT
25 Linker 1 and SLT Linker 2.

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SLT Linker 1 (SEQ ID NO: 75)

5' TGTCATCATCATGCATCGCGAGTTGCCAGAATGGCATCT
GATGAGTTTCCTTCTATGTGCGCAAGTACTC 3'

SLT Linker 2 (SEQ ID NO: 76)

5 TCGAGAGTACTTGCGCACATAGAAGGAACTCATCAGAT
GCCATTCTGGCAACTCGCGATGCATGATGATGACATGCA 3'

The two oligonucleotides were annealed and ligated into a vector containing PstI and XhoI cohesive ends, destroying the PstI site and maintaining the XhoI site. The vector, pING3185, contained an engineered Pst I site at the 3'-end of the Fd gene, and contained an XhoI site downstream of the Fd gene. The product of this ligation, pVK1, contained the H65 Fd gene (fused to the pelB leader) in frame with the SLT linker segment, and contained two restriction sites, FspI and ScaI, at the 3'-end of the SLT linker.

Plasmid pVK1 was digested with SauI and ScaI, and the 217 bp fragment containing a portion of the Fd constant domain and the entire SLT gene segment was purified by electrophoresis on an agarose gel. pING3731 was digested with SmaI and XhoI and the 760 bp gelonin gene was similarly purified. Plasmid pING4000 was digested with SauI and XhoI and the vector segment containing the entire kappa gene and a portion of the Fd gene was also purified. Ligation of these three DNA fragments resulted in pING4406 containing the Fd::SLT::Gelonin (kappa) gene fusion vector.

(2) kappa::SLT::Gelonin (Fd)

A gelonin gene fusion to the 3'-end of the H65 kappa chain with the 25 amino acid SLT linker sequence (20 amino acids from SLT bounded by cysteine residues, plus 5 amino acids introduced to facilitate cloning) was assembled from the DNA segments in pING3731 (ATCC 68721) and pING3713.

Plasmid pING3713 is an Fab expression vector where the H65 Fd and kappa genes are linked in a dicistronic transcription unit containing the SLT linker segment cloned in-frame at the 3'-end of the kappa gene. The plasmid was constructed as follows. In a source plasmid containing the H65 Fd and kappa genes,

5 SLT-Eag-5' (SEQ ID NO: 77)
5' TGTTCCGCCCGCATGTCATCATCATGCATCG 3'
SalI (SEQ ID NO: 78)
5' AGTCATGCCCCGCGC 3'

For construction of gene fusion to gelonin, pING3713 was cut with ScaI and XhoI, and the vector fragment containing the Fd gene and kappa::SLT fusion was purified. pING3731 was digested with SmaI and XhoI and the DNA
15 fragment containing the gelonin gene was also purified. The product of the ligation of these two fragments, pING4407, contains the Fd and kappa::SLT::gelonin genes.

20 A gelonin gene fusion to the 3'-end of the H65 Fd chain with the 21 amino acid RMA linker sequence (20 amino acids from RMA, plus 1 amino acid introduced to facilitate cloning) was assembled in a three piece ligation from plasmids pSH4, pING3731 (ATCC 68721) and pING4000.

Plasmid pSH4 contains an Fd gene linked in frame to the RMA linker sequence. The RMA gene segment was linked to the 3'-end of Fd by overlap extension PCR as follows. The 3'-end (constant region) of the Fd gene was amplified by PCR from a source plasmid with the primers KBA-γ2 and RMAG-1. Any Fd constant region may be used because constant regions of all human IgG₁ antibodies are identical.

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KBA-γ2 (SEQ ID NO: 79)

5' TCCCGGCTGTCCTACAGT 3'

RMAG-1 (SEQ ID NO: 80)

5' TCCAGCCTGTCCAGATGGTGTGTGAGTTTTGTCACAA 3'

5 The product of this reaction was mixed with primer RMA-76, which annealed to the amplified product of the first reaction, and the mixture was amplified with primers KBA-γ2 and RMAK-2.

RMA-76 (SEQ ID NO: 81)

5' CTAAGTCGAGAGTACTGTATGCATGGTTCGAGATGAACA

10 AAGATTCTGAGGCTGCAGCTCCAGCCTGTCCAGATGG 3'

RMAK-2 (SEQ ID NO: 82)

5' CTAAGTCGAGAGTACTGTAT 3'

15 The PCR product contained a portion of the Fd constant region linked in-frame to the RMA gene segment. The product also contained a ScaI restriction site useful for in-frame fusion to a protein such as gelonin, and an XhoI site for subsequent cloning. This PCR product was cut with SauI and XhoI and ligated adjacent to the remainder of the Fd gene to generate pSH4.

20 For assembly of the gene fusion vector containing the Fd::RMA::Gelolin, kappa genes, pSH4 was cut with SauI and ScaI and the Fd::RMA segment was purified. Plasmid pING3731 was cut with SmaI and XhoI and the 760 bp DNA fragment containing the gelolin gene was purified, and pING4000 was cut with SauI and XhoI and the vector was purified. The product of the ligation of these fragments, pING4408, contained the Fd::RMA::Gelolin and kappa genes.

(4) kappa::RMA::Gelolin (Fd)

25 A gelolin gene fusion to the 3'-end of the H65 kappa chain with the 21 amino acid RMA linker sequence was assembled in a three piece ligation from plasmids pSH6, pING4408 (see the foregoing paragraph) and pING3713.

Plasmid pSH6 contains a kappa gene linked in-frame to the RMA linker sequence. The RMA gene segment was linked to the 3'-end of kappa by

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RMAK-1 (SEQ ID NO: 83)

5

5' GTACAGTGGGAAGGTGGAT 3'

10

15

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A gelonin gene fusion to the 5'-end of the H65 Fd chain with a 25 LT linker sequence (20 amino acids from SLT bounded by cystine five amino acids introduced to facilitate cloning) was assembled in a gation from plasmids pING3748, pING3217, and a PCR fragment H65 gamma variable region (V_H) gene segment which is the variable

30

[illegible]

5

gelo3'-Eag (SEQ ID NO: 85)

10

15

H65-G1 (SEQ ID NO: 86)

20

H65-G2 (SEQ ID NO: 87)

25

The 176 bp fragment containing the 5'-end of the H65 heavy chain V-region was purified. Concurrently, pING3217 was digested with NdeI and XhoI, and the 1307 bp DNA fragment containing a portion of the Fd gene and all of the kappa gene was purified. The two fragments were ligated to pING3748 which had been digested with ScaI and XhoI in a three piece ligation yielding pING3754 (ATCC 69102), which contains the Gelonin::SLT::Fd and kappa genes.

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(2) Gelonin::SLT::kappa (Fd)

A gelonin gene fusion to the 5'-end of the H65 kappa chain with the 25 amino acid SLT linker sequence was assembled in a three piece ligation from plasmids pING3748 (see the foregoing section), pING4000, and a PCR fragment encoding the H65 kappa variable region (V_L) gene segment.

For assembly of the gene fusion vector containing the Gelonin::SLT::kappa and Fd genes, an H65 V_L fragment was amplified by PCR from pING3217 with primers H65-K1 and JK1-HindIII, and the product was treated with T4 polymerase followed by digestion with HindIII.

10 H65-K1 (SEQ ID NO: 88)

5' GACATCAAGATGACCCAGT 3'

JK1-HindIII (SEQ ID NO: 89)

5' GTTTGATTTC AAGCTTGGTGC 3'

The 306 bp fragment containing the light chain V-region was purified. Concurrently, pING4000 was digested with HindIII and XhoI, and the 1179 bp DNA fragment containing the kappa constant region and all of the Fd gene was purified. The two fragments were ligated to pING3748 which had been digested with ScaI and XhoI in a three piece ligation yielding pING3757, which contains the Gelonin::SLT::kappa and Fd genes.

20 (3) Gelonin::RMA::Fd (kappa)

A gelonin gene fusion to the 5'-end of the H65 Fd chain with the 24 amino acid RMA linker sequence (20 amino acids from RMA, plus 4 amino acids introduced to facilitate cloning) was assembled in a three piece ligation from plasmids pING3755, pING3217, and a PCR fragment encoding the H65 V_H gene segment. Plasmid pING3755 contains the gelonin gene linked in-frame to the RMA linker sequence, and pING3217 contains the H65 Fd and kappa genes in a dicistronic transcription unit.

Plasmid pING3755 was assembled to contain the gelonin gene linked to the RMA linker gene segment. The RMA linker gene segment was amplified by PCR from pSH4 with primers RMA-EagI and HINDIII-2.

pING3755: RMA::Gel

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RMA-EagI (SEQ ID NO: 90)

5' ACTTCGGCCGCACCATCTGGACAGGCTGGAG 3'

HINDIII-2 (SEQ ID NO: 91)

5' CGTTAGCAATTAACTGTGAT 3'

5 The 198 bp PCR product was cut with EagI and HindIII, and the resulting 153 bp DNA fragment was purified. This RMA gene segment was cloned adjacent to gelonin using an PstI to EagI fragment from pING3748 and the PstI to HindIII vector fragment from pING3825. The product of this three piece ligation was pING3755.

10 For assembly of the gene fusion vector containing the Gelonin::RMA::Fd, kappa genes, the H65 V_H was amplified by PCR from pING3217 with primers H65-G1 (SEQ ID NO: 86) and H65-G2 (SEQ ID NO: 87), and the product was treated with T4 polymerase followed by digestion with NdeI. The 186 bp fragment containing the 5'-end of the heavy chain V-region was purified. Concurrently, pING3217 was digested with NdeI and XhoI, and the 1307 bp DNA
15 fragment containing a portion of the Fd gene and all of the kappa gene was purified. These two fragments were ligated to pING3755 which had been digested with ScaI and XhoI in a three piece ligation yielding pING3759 (ATCC 69104), which contains the Gelonin::RMA::Fd and kappa genes.

(4) Gelonin::RMA::kappa (Fd)

20 A gelonin gene fusion to the 5'-end of the H65 kappa chain with the 24 amino acid RMA linker sequence was assembled in a three piece ligation from plasmids pING3755, pING4000, and a PCR fragment encoding the H65 V_L gene segment.

25 For assembly of the gene fusion vector containing the Gelonin::RMA::kappa and Fd genes, an H65 V_L segment was amplified by PCR from pING3217 with primers H65K-1 (SEQ ID NO: 88) and JK1-HindIII (SEQ ID NO: 89), and the product was treated with T4 polymerase followed by digestion with HindIII. The 306 bp fragment containing the 5'-end of the light chain V-region was purified. Concurrently, pING4000 was digested with HindIII and XhoI, and the 1179
30 bp DNA fragment containing the kappa constant region and all of the Fd gene was

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purified. These two fragments were ligated to pING3755 which had been digested with ScaI and XhoI in a three piece ligation yielding pING3758 (ATCC 69103), which contains the Gelonin::RMA::kappa and Fd genes.

(5) Gelonin::Fd (Kappa)

5 A direct gelonin gene fusion was constructed from pING3754. pING3754 was digested with BglII and XhoI and the vector segment was purified. Concurrently, pING3754 was digested with EagI, treated with T4 polymerase, cut with BglII, and the gelonin gene segment was purified. pING3754 was also cut with FspI and XhoI, and the Fd and kappa gene segment was purified. These fragments
10 were assembled in a three-piece ligation to generate pING3334, which contains a direct gene fusion of gelonin to Fd in association with a kappa gene.

Example 11

Each of the eight gelonin gene fusions whose construction is described in Example 10 was co-expressed with its pair H65 Fab gene in arabinose-induced E. coli strain E104.
15

Expression products of the gene fusions were detected in the supernatant of induced cultures by ELISA. Typically, a plate was coated with antibody recognizing gelonin. Culture supernatant was applied and bound Fab was detected with antibody recognizing human kappa coupled to horseradish peroxidase.
20 H65 Fab fragment chemically conjugated to gelonin was used a standard. Alternative ELISA protocols involving coating a plate with antibody recognizing either the kappa or Fd or involving a detection step with anti-human Fd rather than anti-human kappa yielded similar results. Only properly assembled fusion protein containing gelonin, kappa and Fd was detected by this assay. Unassociated chains were not detected.

25 The fusion protein produced from induced cultures containing expression vectors pING4406, 4407, 4408, and 4410 in E. coli E104 accumulated at about 20-50 ng/ml. The fusion proteins expressed upon induction of pING3754, 3334, 3758 and 3759 (but not pING3757) were expressed at much higher levels, at about 100 to 500 ng/ml. A fusion protein of about 70,000 Kd was detected in the

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concentrated *E. coli* culture supernatant by immunostaining of Western blots with either anti-human kappa or anti-gelonin antibodies.

The Gelonin::SLT::Fd (kappa) fusion protein from pING3754 (ATCC 69102) was purified from induced 10 L fermentation broth. The 10 L fermentation broth was concentrated and buffer exchanged into 10mM phosphate buffer at pH 7.0, using an S10Y10 cartridge (Amicon) and a DC10 concentrator. The supernatant was purified by passing the concentrated supernatant through a DE52 column (20 x 5 cm) equilibrated with 10 mM sodium phosphate buffer at pH 7.0. The flow-through was then further purified and concentrated by column chromatography on CM52 (5 x 10 cm) in 10 mM phosphate buffer. A 0 - 0.2 M linear gradient of NaCl was used to elute the fusion protein, and fractions containing the fusion protein were pooled and loaded onto a Protein G column (1ml). The fusion protein was eluted from protein G with 0.2 M glycine. The Gelonin::RMA::Fd (kappa) and Gelonin::RMA::kappa (Fd) fusion proteins were purified from fermentation broths by similar methods except that the CM52 column step was eliminated, and the DE52 column was equilibrated with 100mM sodium phosphate buffer at pH 7.0. The fusion proteins were not purified to homogeneity.

Each of the three purified fusion proteins was then assayed for activity in the RLA assay and for cytotoxicity against the T-cell line HSB2. (T cells express the CD5 antigen which is recognized by H65 antibody.) The RLA assay was performed as described in Example 4 and results of the assay are presented below in Table 12.

Table 12

	<u>Fusion Protein</u>	<u>IC50(pM)</u>
25	rGelonin	11
	Gelonin::SLT::Fd (kappa)	19
	Gelonin::RMA::Fd (kappa)	28
	Gelonin::RMA::kappa (Fd)	10

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In whole cell cytotoxicity assays performed as described in Example 6, the fusion protein was active and killed two T cell lines, HSB2 and CEM, with respective IC₅₀s 2-fold (HSB2) or 10-fold (CEM) lower than that of the gelonin chemically linked to H65. See Table 13 below for results wherein IC₅₀ values were adjusted relative to the amount of fusion protein in each sample.

Table 13

IC₅₀ (pMT)

	<u>Fusion Protein</u>	<u>IC₅₀ (pMT)</u>	
		<u>HSB2 Cells</u>	<u>CEM Cells</u>
	he3Fab-Gel _{CM}	165	173
10	Gelonin:SLT::Fd + k	180	1007
	Gelonin::RMA::Fd + k	150	nt

The fusion protein showed similar activity on peripheral blood mononuclear cells (data not shown).

Example 12

The natural sequence gelonin gene was also fused to a single chain form of the human engineered he3 H65 variable region. The gelonin gene was positioned at the N-terminus of the fusion gene and the SLT or RMA linker peptide was positioned between the gelonin and antibody domains to allow intracellular processing of the fusion protein with subsequent cytosolic release of gelonin.

A single chain antibody (scAb) form of the he3 H65 variable domain was assembled from previously constructed genes. This scAb segment consisted of the entire V and J region of the one chain (heavy or light) linked to the entire V and J segment of the other chain (heavy or light) via a 15 amino acid flexible peptide: [(Gly)₄ Ser]₃. This peptide is identical to that described in Huston et al., Proc. Natl. Acad. Sci. USA, 85, 5879-5883 (1988); Glockshuber et al., Biochemistry, 29, 1362-1367 (1990); and Cheadle et al., Molecular Immunol., 29, 21-30 (1992). The scAb was assembled in two orientations: V-J_{heavy}::[(Gly)₄ Ser]₃::V-J_{light} (SEQ ID NO: 92)

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and V-J_α::[(Gly)₄Ser]₃::V-J_κ (SEQ ID NO: 93). Each scAb segment was assembled and subsequently fused to gelonin.

For assembly of the scAb segment V-J_κ::[(Gly)₄Ser]₃::V-J_α, primers HUK-7 and SCFV-1 were used to amplify a 352 bp DNA fragment containing the he3 V/J kappa sequences from pING4627 by PCR in a reaction containing 10 mM KCl, 20 mM TRIS pH 8.8, 10 mM (NH₄)₂SO₄, 2mM MgSO₄, 0.1% Triton X-100., 100 ng/ml BSA, 200 μM of each dNTP, and 2 Units of Vent polymerase (New England Biolabs, Beverly, Massachusetts) in a total volume of 100 μl.

SCFV-1 (SEQ ID NO:94)

5' CGGACCCACCTCCACCAGATCCACCGC
CACCTTTCATCTCAAGCTTGGTGC 3'

HUK-7 (SEQ ID NO: 95)

5' GACATCCAGATGACTCAGT 3'

Concurrently, primers SCFV-2 and SCFV-3 were used to amplify a he3 heavy chain V/J gamma segment from pING4623, generating a 400 bp fragment.

SCFV-2 (SEQ ID NO: 96)

5' GGTGGAGGTGGGTCCGGAGGTGGAGGATCTGA
GATCCAGTTGGTGCAGT 3'

SCFV-3 (SEQ ID NO: 97)

5' TGTACTCGAGCCCATCATGAGGAGACGGTGACCGT 3'

The products from these reactions were mixed and amplified with the outside primers HUK-7 and SCFV-3. The product of this reaction was treated with T4 polymerase and then cut with XhoI. The resulting 728 bp fragment was then purified by electrophoresis on an agarose gel. This fragment was ligated into the vectors pING3755 and pING3748 (see Example 10), each digested with ScaI and XhoI. The resulting vectors pING4637 and pING4412 contain the Gelonin::RMA::scab V-J_κ::[(Gly)₄Ser]₃::V-J_α and Gelonin::SLT::scAb V-J_κ::[(Gly)₄Ser]₃::V-J_α fusion genes respectively.

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Similarly, the scAb V-J_α::[(Gly)₄Ser]₃::V-J_κ was assembled by amplification of pING4627 with primers SCFV-5 and SCFV-6 generating a 367 bp fragment containing he3 V/J kappa sequences,

SCFV-5 (SEQ ID NO: 98)

5' GGTGGAGGTGGGTCCGGAGGTGGAGGATCT 3'

SCFV-6 (SEQ ID NO: 99)

5' TGTACTCGAGCCCATCATTTTCATCTCAAGCTTGGTGC 3'

and pING4623 with primers H65-G3 and SCFV-4 generating a 385 bp fragment containing he3 gamma V/J sequences by PCR with Vent polymerase.

H65-G3 (SEQ ID NO: 100)

5' GAGATCCAGTTGGTGCACTCTG 3'

SCFV-4 (SEQ ID NO: 101)

5' CGGACCCACCTCCACCAGATCC

ACCGCCACCTGAGGAGACGGTGACCGT 3'

The products from these reactions were mixed and amplified with H65-G3 and SCFV-6. The 737 bp product was treated with T4 polymerase and cut with XhoI. Ligation into pING3755 and pING3748 (digested with ScaI and XhoI) results in assembly of the Gelonin::RMA::scAb V-J_α::[(Gly)₄Ser]₃::V-J_κ and Gelonin::SLT::scAb V-J_α::[(Gly)₄Ser]₃::V-J_κ fusion genes, respectively.

Gelonin::scAb fusions without a cleavable linker can be constructed by deletion of the SLT linker in pING4412 using the restriction enzymes EagI and FspI. Digestion at these sites and religation of the plasmid results in an in-frame deletion of the SLT sequence.

EXAMPLE 13

BRIP possesses characteristics which make it an attractive candidate for a component of immunotoxins. BRIP is a naturally unglycosylated protein that may have reduced uptake in the liver and enhanced circulatory residence time *in vivo*. Additionally, BRIP is less toxic and less immunogenic in animals than the A-chain of ricin. Cloning of the BRIP gene and expression of recombinant BRIP in an *E. coli*

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expression system obviates the need to purify native BRIP directly from barley, and enables the development of analogs of BRIP which may be conjugated with an available cysteine residue for conjugation to antibodies.

Purification of BRIP and Generation
of Polyclonal Antibodies to BRIP

Native BRIP was purified from pearled barley flour. Four kilograms of flour was extracted with 16 liters of extraction buffer (10 mM NaPO₄, 25 mM NaCl, pH 7.2) for 20 hours at 4°C. The sediment was removed by centrifugation, and 200 ml of packed S-Sepharose (Pharmacia, Piscataway, New Jersey) was added to absorb BRIP. After mixing for 20 hours at 4°C, the resin was allowed to settle out, rinsed several times with extraction buffer and then packed into a 2.6 x 40 cm column. Once packed, the column was washed with extraction buffer (150 ml/h) until the absorbance of the effluent approached zero. BRIP was then eluted with a linear gradient of 0.025 to 0.3 M NaCl in extraction buffer and 5 ml fractions were collected. BRIP-containing peaks (identified by Western analysis of column fractions) were pooled, concentrated to about 20 ml, and then chromatographed on a 2.6 x 100 cm Sephacryl S-200HR (Pharmacia) column equilibrated in 10 mM NaPO₄, 125 mM NaCl, pH 7.4 (10 ml/hr). BRIP-containing peaks were pooled again, concentrated, and stored at -70°C.

The resulting purified BRIP protein had a molecular weight of about 30,000 Daltons, based upon the mobility of Coomassie-stained protein bands following SDS-PAGE. The amino acid composition was consistent with that published by Asano et al., Carlsberg Res. Comm., 49, 619-626 (1984).

Rabbits were immunized with purified BRIP to generate polyclonal antisera.

Cloning of the BRIP Gene

A cDNA expression library prepared from germinating barley seeds in the phage λ expression vector λ ZAPII was purchased from Stratagene, La Jolla, CA. Approximately 700,000 phage plaques were screened with anti-BRIP polyclonal antisera and 6 immunoreactive plaques were identified. One plaque was chosen, and

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the cDNA contained therein was excised from λ ZAPII with EcoRI and subcloned into pUC18 generating the vector pBS1. The cDNA insert was sequenced with Sequenase (United States Biochemical, Cleveland, Ohio). The DNA sequence of the native BRIP gene is set out in SEQ ID NO: 12. To confirm that cDNA encoded the native
 5 BRIP gene, the cDNA was expressed in the *E. coli* plasmid pKK233-2 (Pharmacia). BRIP protein was detected in IPTG-induced cells transformed with the plasmid by Western analysis with above-described rabbit anti-BRIP antisera.

Construction of an *E. coli* Expression

Vector Containing the BRIP Gene

10 Barley cDNA containing the BRIP gene was linked to a *pe*_B leader sequence and placed under control of an *ara*B promoter in a bacterial secretion vector.

An intermediate vector containing the BRIP gene linked to the *pe*_B leader sequence was generated. Plasmid pBS1 was cut with NcoI, treated with Mung Bean Nuclease, cut with BamHI and the 760 bp fragment corresponding to amino
 15 acids 1-256 of BRIP was purified from an agarose gel. Concurrently, a unique XhoI site was introduced downstream of the 3'-end of the BRIP gene in pBS1 by PCR amplification with a pUC18 vector primer (identical to the Reverse[®] primer sold by NEB or BRL but synthesized on a Cyclone Model 8400 DNA synthesizer) and the specific primer BRIP 3'Xho. The sequence of each of the primers is set out below.

20 Reverse (SEQ ID NO: 45)

5' AACAGCTATGACCATG 3'

BRIP 3'Xho (SEQ ID NO: 46)

5' TGAAGTCGAGGAAAACCTACCTATTTCCCAC 3'

25 Primer BRIP 3'Xho includes a portion corresponding to the last 8 bp of the BRIP gene, the termination codon and several base pairs downstream of the BRIP gene, and an additional portion that introduces a XhoI site in the resulting PCR fragment. The PCR reaction product was digested with BamHI and XhoI, and an 87 bp fragment containing the 3'-end of the BRIP gene was purified on a 5% acrylamide gel. The 760 and 87 bp purified BRIP fragments were ligated in the vector pING1500 adjacent
 30 to the *pe*_B leader sequence. pING1500 had previously been cut with SstI, treated

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with T4 polymerase, cut with XhoI, and purified. The DNA sequence at the junction of the *pelB* leader and the 5'-end of the BRIP gene was verified by DNA sequence analysis. This vector was denoted pING3321-1.

5 The final expression vector was assembled by placing the BRIP gene under the control of the inducible *araB* promoter. Plasmid pING3321-1 was cut with PstI and XhoI, and the BRIP gene linked to the *pelB* leader was purified from an agarose gel. The expression vector pING3217, containing the *araB* promoter, was cut with PstI and XhoI and ligated to the BRIP gene. The expression vector was denoted pING3322.

10 Arabinose induction of *E. coli* cells containing the plasmid pING3322 in a fermenter resulted in the production of about 100 mg per liter of recombinant BRIP. *E. coli*-produced BRIP displays properties identical to BRIP purified directly from barley seeds.

Construction of BRIP Analogs

15 With a Free Cysteine Residue

The BRIP protein contains no cysteine residues, and therefore contains no residues directly available which may form a disulfide linkage to antibodies or other proteins. Analogs of recombinant BRIP were generated which contain a free cysteine residue near the C-terminus of the protein. Three residues of the BRIP
20 protein were targets for amino acid substitutions. Comparison of the amino acid sequence of BRIP to the known tertiary structure of the ricin A-chain (see FIG. 2) suggested that the three positions would be available near the surface of the molecule. The three BRIP analogs include cysteines substituted in place of serine₂₇₇, alanine₂₇₀, and leucine₂₃₆ of the native protein, and were designated BRIP_{C277}, BRIP_{C270} and
25 BRIP_{C236}, respectively.

(1) A plasmid vector capable of expressing the BRIP_{C277} analog was constructed by replacing the 3'-end of the BRIP gene with a DNA segment conferring the amino acid change. The EcoRI fragment containing the BRIP gene from pBS1 was subcloned into M13mp18, and single-stranded DNA (anti-sense strand) was
30 amplified by PCR with primers OBM2 (corresponding nucleotides -11 to +8 of the

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BRIP gene) and OMB4 (corresponding to amino acids 264-280 of BRIP and the termination codon of BRIP, and incorporating the substitution of a cysteine codon for the native codon for serine₂₇₇ of native BRIP). The sequences of primers OBM2 and OMB4, wherein the underlined nucleotides encode the substituted cysteine, are set out below.

OBM2 (SEQ ID NO: 47)

5' GCATTACATCCATGGCGGC 3'

OMB4 (SEQ ID NO: 48)

5' GATATCTCGAGTTAACTATTTCCCACCAACACG
CATGGAACAGCTCCAGCGCCTTGGCCACCGTC 3'

A fragment containing a BRIP gene in which the codon for the amino acid at position 277 was changed to a cysteine codon was amplified. The fragment was cloned into the SmaI site of pUC19 (BRL) and the plasmid generated was denoted pMB22. pMB22 was digested with EcoRI and an EcoRI-XhoI linker (Clontech, Palo Alto, CA) was ligated into the vector. Subsequent digestion with XhoI and religation generated vector pINGMB2X. A BamHI to XhoI fragment encoding the 3'-end of BRIP with the altered amino acid was excised from pMB2X and the fragment was purified on a 5% acrylamide gel. This fragment along with an EcoRI to BamHI fragment containing the *pelB* leader sequence and sequences encoding the first 256 amino acids of BRIP were substituted in a three piece ligation into pING3322 cut with EcoRI and XhoI. The resulting vector containing the BRIP_{CTM} analog was designated pING3803 (ATCC Accession No. 68722).

(2) A BRIP analog with a free cysteine at position 256 was constructed using PCR to introduce the amino acid substitution. A portion of the expression plasmid pING3322 was amplified with primers BRIP-256 and HINDIII-2. The sequence of each primer is set out below.

BRIP-256 (SEQ ID NO: 49)

5' TGTCTGTTCGTGGAGGTGCCG 3'

HINDIII-2 (SEQ ID NO: 50)

5' CGTTAGCAATTAACTGTGAT 3'

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Nucleotides 4-21 of primer BRIP-256 encode amino acids 256-262 of BRIP while the underlined nucleotides specify the cysteine to be substituted for the leucine at the corresponding position of the native BRIP protein. Primer HINDIII-2 corresponds to a portion of the plasmid. The PCR product, which encodes the carboxyl terminal portion of the BRIP analog, was treated with T4 polymerase, cut with XhoI, and the resulting fragment was purified on a 5% acrylamide gel. Concurrently, plasmid pING3322 was cut with BamHI, treated with T4 polymerase, cut with EcoRI, and the fragment containing the *pelB* leader sequence and sequences encoding the first 256 amino acids of BRIP was purified. The two fragments were then assembled back into pING3322 to generate the gene encoding the analog BRIP_{C256}. This plasmid is denoted pING3801.

(3) A BRIP analog with a cysteine at position 270 was also generated using PCR. A portion of the expression plasmid pING3322 was amplified with primers BRIP-270 and the HINDIII-2 primer (SEQ ID NO: 50). The sequence of primer BRIP-270 is set out below.

BRIP-270 (SEQ ID NO: 51)

5' CCAAGTGTCTGGAGCTGTTCCATGCGA 3'

Primer BRIP-270 corresponds to amino acids 268-276 of BRIP with the exception of residue 270. The codon of the primer corresponding to position 270 specifies a cysteine instead of the alanine present in the corresponding position in native BRIP. The PCR product was treated with T4 polymerase, cut with XhoI, and the 51 bp fragment, which encodes the carboxyl terminal portion of the analog, was purified on a 5% acrylamide gel. The fragment (corresponding to amino acids 268-276 of BRIP_{C270}) was cloned in a three piece ligation along with the internal 151 bp BRIP restriction fragment from SstII to MscI (corresponding to BRIP amino acids 217-267) from plasmid pING3322, and restriction fragment from SstII to XhoI from pING3322 containing the remainder of the BRIP gene. The plasmid generated contains the gene encoding the BRIP_{C270} analog and is designated pING3802.

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Purification of Recombinant BRIP
and the BRIP Analogs

Recombinant BRIP (rBRIP) and the BRIP analogs with free cysteine residues were purified essentially as described for native BRIP except they were prepared from concentrated fermentation broths. For rBRIP, concentrated broth from a 10 liter fermentation batch was exchanged into 10 mM Tris, 20 mM NaCl pH 7.5, loaded onto a Sephacryl S-200 column, and eluted with a 20 to 500 mM NaCl linear gradient. Pooled rBRIP was further purified on a Blue Toyopearl® column (TosoHaas) loaded in 20 mM NaCl and eluted in a 20 to 500 mM NaCl gradient in 10mM Tris, pH 7.5. For BRIP analogs, concentrated fermentation broths were loaded onto a CM52 column (Whatman) in 10 mM phosphate buffer, pH 7.5, and eluted with a 0 to 0.3M NaCl linear gradient. Further purification was by chromatography on a Blue Toyopearl® column.

Reticulocyte Lysate Assay

The ability of the rBRIP and the BRIP analogs to inhibit protein synthesis *in vitro* was tested by reticulocyte lysate assay as described in Example 1. Serial log dilutions of standard toxin (RTA 30), native BRIP, rBRIP and BRIP analogs were tested over a range of 1 µg/ml to 1 pg/ml. By comparison with an uninhibited sample, the picomolar concentration of toxin (pM) which corresponds to 50% inhibition of protein synthesis (IC₅₀) was calculated. The results of the assays are presented below in Table 14.

Table 14

	<u>Toxin</u>	<u>IC₅₀ (pM)</u>
	RTA 30	3.1
25	Native BRIP	15
	rBRIP	18
	BRIP _{C256}	23
	BRIP _{C770}	20
	BRIP _{C777}	24

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The RLA results indicate that the BRIP analogs exhibit ribosome-inactivating activity comparable to that of the recombinant and native BRIP toxin. All the analogs retained the natural ability of native BRIP to inhibit protein synthesis, suggesting that amino acid substitution at these positions does not affect protein folding and activity.

Construction of BRIP Immunoconjugates

Immunoconjugates of native BRIP with 4A2 (described in Morishima et al., *J. Immunol.*, 129, 1091 (1982) and H65 antibody (obtained from hybridoma ATCC HB9286) which recognize the T-cell determinants CD7 and CD5, respectively, were constructed. Immunoconjugates of ricin A-chains (RTAs) with 4A2 and H65 antibody were constructed as controls. The H65 antibody and ricin A-chains as well as the RTA immunoconjugates were prepared and purified according to methods described in U.S. Patent Application Serial No. 07/306,433 *supra* and in International Publication No. WO 89/06968.

To prepare immunoconjugates of native BRIP, both the antibody (4A2 or H65) and native BRIP were chemically modified with the hindered linker 5-methyl-2-iminothiolane (M2IT) at lysine residues to introduce a reactive sulfhydryl group as described in Goff et al., *Bioconjugate Chem.*, 1, 381-386 (1990). BRIP (3 mg/ml) was first incubated with 0.5 mM M2IT and 1 mM DTNB in 25 mM triethanolamine, 150 mM NaCl, pH 8.0, for 3 hours at 25°C. The derivitized BRIP-(M2IT)-S-S-TNB was then desalted on a column of Sephadex GF-05LS and the number of thiol groups introduced was quantitated by the addition of 0.1 mM DTT. On average, each BRIP molecule contained 0.7 SH/mol.

4A2 or H65 antibody (4 mg/ml) in triethanolamine buffer was similarly incubated with M2IT (0.3 mM) and DTNB (1 mM) for 3 hours at 25°C. Antibody-(M2IT)-S-S-TNB was then desalted and the TNB:antibody ratio was determined. To prepare the conjugate, the BRIP-(M2IT)-S-S-TNB was first reduced to BRIP-(M2IT)-SH by treatment with 0.5 mM DTT for 1 hour at 25°C, desalted by gel filtration of Sephadex® GF-05LS to remove the reducing agent, and then mixed with antibody-(M2IT)-S-S-TNB.

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Following a 3 hour incubation at 25°C, and an additional 18 hours at 4°C, the conjugate was purified by sequential chromatography on AcA44 (IBF) and Blue Toyopearl®. Samples of the final product were run on 5% non-reducing SDS PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody.

The BRIP analogs containing a free cysteine were also conjugated to 4A2 and H65 antibodies. The analogs were treated with 50 mM DTT either for 2 hours at 25°C or for 18 hours at 4°C to expose the reactive sulfhydryl group of the cysteine and desalted. The presence of a free sulfhydryl was verified by reaction with DTNB [Ellman et al., *Arch. Biochem. Biophys.*, 82, 70-77 (1959)]. 4A2 or H65 antibody derivatized as described above with M2IT was incubated with the reduced BRIP analogs at a ratio of 1:5 at room temperature for 3 hours and then overnight at 4°C. Immunoconjugates H65-BRIP_{C256}, 4A2-BRIP_{C256}, H65-BRIP_{C77} were prepared in 25 mM triethanolamine, 150 mM NaCl pH 8, while immunoconjugates H65-BRIP_{C770}, 4A2-BRIP_{C770} and 4A2-BRIP_{C77} were prepared in 0.1 M sodium phosphate, 150 mM NaCl pH 7.5. Following conjugation, 10 µM mercaptoethylamine was added for 15 minutes at 25°C to quenched any unreacted m2IT linkers on the antibody. The quenched reaction solution was promptly loaded onto a gel filtration column (AcA44) to remove unconjugated ribosome-inactivating protein. Purification was completed using soft gel affinity chromatography on Blue Toyopearl® resin using a method similar to Knowles et al., *Analyt. Biochem.*, 160, 440 (1987). Samples of the final product were run on 5% non-reduced SDS PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody. The conjugation efficiency was substantially greater for BRIP_{C77} (78%) than for either of the other two analogs, BRIP_{C770} and BRIP_{C256} (each of these was about 10%). Additionally, the BRIP_{C77} product was a polyconjugate, i.e., several BRIP molecules conjugated to a single antibody, in contrast to the BRIP_{C770} and BRIP_{C256} products which were monoconjugates.

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Whole Cell Kill Assay

Immunconjugates of native BRIP and of the BRIP analogs were tested for the ability to inhibit protein synthesis in HSB2 cells by the whole cell kill assay described in Example 1. Standard immunconjugates H65-RTA (H65 derivatized with SPDP linked to RTA) and 4MRTA (4A2 antibody derivatized with M2IT linked to RTA) and BRIP immunconjugate samples were diluted with RPMI without leucine at half-log concentrations ranging from 2000 to 0.632 ng/ml. All dilutions were added in triplicate to microtiter plates containing 1×10^5 HSB2 cells. HSB2 plates were incubated for 20 hours at 37°C and then pulsed with ^3H -Leu for 4 hours before harvesting. Samples were counted on the Inotec Trace 96 cascade ionization counter. By comparison with an untreated sample, the picomolar toxin concentration (pM T) of immunconjugate which resulted in a 50% inhibition of protein synthesis (IC_{50}) was calculated. The assay results are presented below in Table 15.

Table 15

	Conjugate	IC_{50} (pM T)
15	4A2-BRIP	122.45
	4A2-BRIP _{C770}	46.3
	4A2-BRIP _{C277}	57.5
	4A2-BRIP _{C236}	1116
20	H65-BRIP	> 5000
	H65-BRIP _{C277}	1176

The BRIP analog conjugates were less potent than the ricin conjugate control (data not shown). The immunotoxins containing antibody 4A2 and either the BRIP_{C770} or the BRIP_{C277} analog exhibited comparable to increased specific cytotoxicity toward target cells as compared to immunotoxin containing native BRIP. While 4A2-BRIP_{C236} is less active than 4A2-BRIP, 4A2-BRIP_{C770} and 4A2-BRIP_{C277} are between 3 and 4 times more active. Similarly, the immunconjugate of H65 to BRIP_{C277} shows greater toxicity toward target cells than the immunconjugate of H65

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to native BRIP. Thus, linkage of antibody to BRIP derivatives which have an available cysteine residue in an appropriate location results in immunotoxins with enhanced specific toxicity toward target cells relative to conjugates with native BRIP.

Disulfide Bond Stability Assay

5 Immunoconjugates prepared with native BRIP and the BRIP analogs were examined by the disulfide bond stability assay described in Example 1. Briefly, conjugates were incubated with increasing concentrations of glutathione for 1 hour at 37°C and, after terminating the reaction with iodoacetamide, the amount of RIP released was quantitated by size-exclusion HPLC on a TosoHaas TSK-G2000SW
10 column.

By comparisons with the amount of RIP released by high concentrations of 2-mercaptoethanol (to determine 100% release), the concentration of glutathione required to release 50% of the RIP (the RC_{50}) was calculated. As shown below in Table 16, the conjugates prepared with BRIP_{C270} or BRIP_{C277} were
15 significantly more stable than either the RTA conjugates or those prepared with native BRIP.

Table 16

	<u>Conjugate</u>	<u>RC₅₀ (mM)</u>
	H65-RTA	7.0
20	H65-BRIP	2.8
	H65-BRIPC277	196.0
	4A2-RTA	4.4
	4A2-BRIP	3.3
	4A2-BRIP _{C270}	53.0
25	4A2-BRIP _{C277}	187.0

These unexpected results suggest that conjugates prepared with Type I RIP analogs according to the present invention may have enhanced stability and efficacy in vivo.

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EXAMPLE 14

Plants of the genus Momordica produce a number of related proteins known as momordins or momorcharins which are Type I RIPs. The gene encoding momordin II was cloned from Momordica balsamina seeds.

5 Preparation of *M. balsamina* RNA

10 Total RNA was prepared from 4 g of *M. balsamina* seeds as described in Ausubel et al., supra. PolyA containing RNA was prepared from 1 mg of total RNA by chromatography on oligo-(dT)-cellulose. 40 mg of oligo-(dT)-cellulose Type 7 (Pharmacia) was added to 0.1 N NaOH and poured into a disposable column (Biorad). The column was washed with water until the eluate was pH 5.5, and then was washed with 1X loading buffer (50 mM NaCitrate, 0.5M NaCl, 1 mM EDTA, 0.1% SDS, pH 7.0) until the eluate was pH 7.0. 1 mg of total RNA was suspended in 300 µl of water, heated to 65°C for 5 minutes, and 300 µl of 2X loading buffer was added (100 mM Na Citrate, 1M NaCl, 2 mM EDTA, and 0.2% SDS). The RNA was loaded onto the column, and the flow through was reheated to 65°C, cooled to room temperature, and reloaded onto the column. Column-bound mRNA was washed 5 times with 0.5 ml of 1X loading buffer, and two times with 0.5 ml of 0.05M NaCitrate, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS. PolyA- containing RNA was eluted two times from the column with 0.5 ml of 25 mM NaCitrate, 1 mM EDTA, and 0.05% SDS.

15 Library Preparation

20 A cDNA library from the polyA-containing *M. balsamina* RNA was prepared in a bacterial expression plasmid with the SuperScript Plasmid System (BRL, Gaithersburg, Maryland). The cDNA was synthesized from 2 µg of poly A-containing RNA, size fractionated, digested with NotI, and ligated into the expression vector pSPORT as recommended by the manufacturer of the vector, BRL.

25 Cloning of the Momordin II Gene

30 A DNA fragment encoding the first 27 amino acids of momordin II was amplified from *M. balsamina* cDNA by PCR. First strand cDNA was prepared from 100 ng of polyA containing RNA with an RNA-PCR Kit (Perkin Elmer Cetus).

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Primer momo-5 corresponds to amino acids 9-18 of mature momordin II. The underlined nucleotides of the primer were expected to match the DNA sequence of the momordin II gene exactly. Since this sequence is highly A/T-rich and may hybridize to the momordin II gene weakly, the additional adjacent nucleotides were included in the primer. Bases 3 and 30 (overlined) were in the "wobble" position (i.e., the third nucleotide in a codon) of amino acids 9 (alanine) and 18 (isoleucine), respectively, of momordin II and may not be identical to the nucleotide bases in the native gene.

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A 90,000 member cDNA library in pSPORT was screened with ³²P-kinased momo-5, and eight potential candidate clones were identified. One clone, pING3619, was sequenced and contains an open reading frame corresponding in part to the expected N-terminal 27 residues of Momordin II. The complete momordin gene contains 286 amino acids, the first 23 of which are a presumed leader signal (mature momordin II is 263 residues). The DNA sequence of the momordin II gene is set out in SEQ ID NO: 13.

Construction of an Expression Vector
Containing the Momordin II Gene

A bacterial expression vector for the momordin II gene was constructed. Two PCR primers were synthesized, one (momo-9) which primes from the +1 residue of the mature momordin II amino acid sequence, and one at the C-terminus (momo-10) of momordin II which introduces an XhoI restriction site:

momo-9 (SEQ ID NO: 55)

5' GATGTAACTTCGATTGTCTGA 3'

momo-10 (SEQ ID NO: 56)

5' TCAACTCGAGGTACTCAATTCACAACAGATTCC 3'

pING3619 was amplified with momo-9 and momo-10, and the product was treated with T4 polymerase, cut with XhoI, and purified on an agarose gel. This gene fragment was ligated along with the 131 bp pelB leader fragment from pIC100 which has been generated by SstI digestion, T4-polymerase treatment, and EcoRI digestion, into the araB expression vector cleaved with EcoRI and XhoI. The product of this three piece ligation was sequenced to verify that the pelB junction and momordin II coding sequence were correct. Arabinose induction of cells containing the momordin II expression plasmid pING3621 results in production of momordin II in E. coli.

Analog of Momordin II

Momordin II has no natural cysteines available for conjugation to antibody. Analog of momordin which have a free cysteine for conjugation to an antibody may be constructed. Positions likely to be appropriate for substitution of a cysteine residue may be identified from Figure 3 as positions near the ricin A-chain

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cysteine₂₅₉ and as positions including the last 26 amino acids of momordin II that are accessible to solvent. For example, the arginine at position 242 of momordin II aligns with the ricin A-chain cysteine at position 259 and is a preferred target for substitution. Additional preferred substitution positions for momordin II include the serine at position 241 and the alanine at position 243.

While the present invention has been described in terms of preferred embodiments, it is understood that variations and improvements will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Bernhard, Susan L.
Better, Marc D.
Carroll, Stephen F.
Lane, Julie A.
Lei, Shau-Ping
- (ii) TITLE OF INVENTION: Materials Comprising and Methods of Preparation and Use for Ribosome-Inactivating Proteins
- (iii) NUMBER OF SEQUENCES: 101
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Bicknell
(B) STREET: Two First National Plaza, 20 South Clark Street
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60603
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/901,707
(B) FILING DATE: 19-JUN-1992
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/787,567
(B) FILING DATE: 04-NOV-1991
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(C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

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[illegible]

Ile	Phe	Pro	Lys	Gln	Tyr	Pro	Ile	Ile	Asn	Phe	Thr	Thr	Ala	Gly	Ala
1				5						10				15	
Thr	Val	Gln	Ser	Tyr	Thr	Asn	Phe	Ile	Arg	Ala	Val	Arg	Gly	Arg	Leu
		20						25					30		
Thr	Thr	Gly	Ala	Asp	Val	Arg	His	Glu	Ile	Pro	Val	Leu	Pro	Asn	Arg
		35					40					45			
Val	Gly	Leu	Pro	Ile	Asn	Gln	Arg	Phe	Ile	Leu	Val	Glu	Leu	Ser	Asn
	50					55					60				
His	Ala	Glu	Leu	Ser	Val	Thr	Leu	Ala	Leu	Asp	Val	Thr	Asn	Ala	Tyr
65					70					75					80
Val	Val	Gly	Tyr	Arg	Ala	Gly	Asn	Ser	Ala	Tyr	Phe	Phe	His	Pro	Asp
				85					90					95	
Asn	Gln	Glu	Asp	Ala	Glu	Ala	Ile	Thr	His	Leu	Phe	Thr	Asp	Val	Gln
			100					105					110		
Asn	Arg	Tyr	Thr	Phe	Ala	Phe	Gly	Gly	Asn	Tyr	Asp	Arg	Leu	Glu	Gln
		115					120					125			
Leu	Ala	Gly	Asn	Leu	Arg	Glu	Asn	Ile	Glu	Leu	Gly	Asn	Gly	Pro	Leu
	130					135					140				
Glu	Glu	Ala	Ile	Ser	Ala	Leu	Tyr	Tyr	Tyr	Ser	Thr	Gly	Gly	Thr	Gln
145					150					155					160
Leu	Pro	Thr	Leu	Ala	Arg	Ser	Phe	Ile	Ile	Cys	Ile	Gln	Met	Ile	Ser
				165					170					175	
Glu	Ala	Ala	Arg	Phe	Gln	Tyr	Ile	Glu	Gly	Glu	Met	Arg	Thr	Arg	Ile
			180					185					190		
Arg	Tyr	Asn	Arg	Arg	Ser	Ala	Pro	Asp	Pro	Ser	Val	Ile	Thr	Leu	Glu
		195					200					205			
Asn	Ser	Trp	Gly	Arg	Leu	Ser	Thr	Ala	Ile	Gln	Glu	Ser	Asn	Gln	Gly
	210					215					220				
Ala	Phe	Ala	Ser	Pro	Ile	Gln	Leu	Gln	Arg	Arg	Asn	Gly	Ser	Lys	Phe
225					230					235					240
Ser	Val	Tyr	Asp	Val	Ser	Ile	Leu	Ile	Pro	Ile	Ile	Ala	Leu	Met	Val
				245					250					255	
Tyr	Arg	Cys	Ala	Pro	Pro	Pro	Ser	Ser	Gln	Phe					
			260					265							

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 251 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr
 1 5 10 15
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly
 20 25 30
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly
 35 40 45
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala
 50 55 60
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val
 65 70 75 80
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu
 85 90 95
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Thr
 100 105 110
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu
 115 120 125
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala
 130 135 140
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val
 145 150 155 160
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln
 165 170 175
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile
 180 185 190
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser
 195 200 205
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn
 210 215 220
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile
 225 230 235 240
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys
 245 250

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 280 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Lys Met Ala Lys Asn Val Asp Lys Pro Leu Phe Thr Ala Thr
 1 5 10 15

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Phe Asn Val Gln Ala Ser Ser Ala Asp Tyr Ala Thr Phe Ile Ala Gly
 20 25 30
 Ile Arg Asn Lys Leu Arg Asn Pro Ala His Phe Ser His Asn Arg Pro
 35 40 45
 Val Leu Pro Pro Val Glu Pro Asn Val Pro Pro Ser Arg Trp Phe His
 50 55 60
 Val Val Leu Lys Ala Ser Pro Thr Ser Ala Gly Leu Thr Leu Ala Ile
 65 70 75 80
 Arg Ala Asp Asn Ile Tyr Leu Glu Gly Phe Lys Ser Ser Asp Gly Thr
 85 90 95
 Trp Trp Glu Leu Thr Pro Gly Leu Ile Pro Gly Ala Thr Tyr Val Gly
 100 105 110
 Phe Gly Gly Thr Tyr Arg Asp Leu Leu Gly Asp Thr Asp Lys Leu Thr
 115 120 125
 Asn Val Ala Leu Gly Arg Gln Gln Leu Ala Asp Ala Val Thr Ala Leu
 130 135 140
 His Gly Arg Thr Lys Ala Asp Lys Ala Ser Gly Pro Lys Gln Gln Gln
 145 150 155 160
 Ala Arg Glu Ala Val Thr Thr Leu Val Leu Met Val Asn Glu Ala Thr
 165 170 175
 Arg Phe Gln Thr Val Ser Gly Phe Val Ala Gly Leu Leu His Pro Lys
 180 185 190
 Ala Val Glu Lys Lys Ser Gly Lys Ile Gly Asn Glu Met Lys Ala Gln
 195 200 205
 Val Asn Gly Trp Gln Asp Leu Ser Ala Ala Leu Leu Lys Thr Asp Val
 210 215 220
 Lys Pro Pro Pro Gly Lys Ser Pro Ala Lys Phe Ala Pro Ile Glu Lys
 225 230 235 240
 Met Gly Val Arg Thr Ala Glu Gln Ala Ala Asn Thr Leu Gly Ile Leu
 245 250 255
 Leu Phe Val Glu Val Pro Gly Gly Leu Thr Val Ala Lys Ala Leu Glu
 260 265 270
 Leu Phe His Ala Ser Gly Gly Lys
 275 280

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 263 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Val Asn Phe Asp Leu Ser Thr Ala Thr Ala Lys Thr Tyr Thr Lys
 1 5 10 15

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Phe Ile Glu Asp Phe Arg Ala Thr Leu Pro Phe Ser His Lys Val Tyr
 20 25 30
 Asp Ile Pro Leu Leu Tyr Ser Thr Ile Ser Asp Ser Arg Arg Phe Ile
 35 40 45
 Leu Leu Asp Leu Thr Ser Tyr Ala Tyr Glu Thr Ile Ser Val Ala Ile
 50 55 60
 Asp Val Thr Asn Val Tyr Val Val Ala Tyr Arg Thr Arg Asp Val Ser
 65 70 75 80
 Tyr Phe Phe Lys Glu Ser Pro Pro Glu Ala Tyr Asn Ile Leu Phe Lys
 85 90 95
 Gly Thr Arg Lys Ile Thr Leu Pro Tyr Thr Gly Asn Tyr Glu Asn Leu
 100 105 110
 Gln Thr Ala Ala His Lys Ile Arg Glu Asn Ile Asp Leu Gly Leu Pro
 115 120 125
 Ala Leu Ser Ser Ala Ile Thr Thr Leu Phe Tyr Tyr Asn Ala Gln Ser
 130 135 140
 Ala Pro Ser Ala Leu Leu Val Leu Ile Gln Thr Thr Ala Glu Ala Ala
 145 150 155 160
 Arg Phe Lys Tyr Ile Glu Arg His Val Ala Lys Tyr Val Ala Thr Asn
 165 170 175
 Phe Lys Pro Asn Leu Ala Ile Ile Ser Leu Glu Asn Gln Trp Ser Ala
 180 185 190
 Leu Ser Lys Gln Ile Phe Leu Ala Gln Asn Gln Gly Gly Lys Phe Arg
 195 200 205
 Asn Pro Val Asp Leu Ile Lys Pro Thr Gly Glu Arg Phe Gln Val Thr
 210 215 220
 Asn Val Asp Ser Asp Val Val Lys Gly Asn Ile Lys Leu Leu Leu Asn
 225 230 235 240
 Ser Arg Ala Ser Thr Ala Asp Glu Asn Phe Ile Thr Thr Met Thr Leu
 245 250 255
 Leu Gly Glu Ser Val Val Asn
 260

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 248 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Val Arg Phe Ser Leu Ser Gly Ser Ser Ser Thr Ser Tyr Ser Lys
 1 5 10 15
 Phe Ile Gly Asp Leu Arg Lys Ala Leu Pro Ser Asn Gly Thr Val Tyr
 20 25 30

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Variable	Mean	SD	Min	Max
Age	35.2	12.5	18	65
Gender	0.52	0.50	0	1
Marital status	0.65	0.48	0	1
Education	12.8	2.1	9	16
Income	15.2	8.5	5	35
Occupation	1.2	0.8	0	2
Health status	0.75	0.42	0	1
Stress level	2.1	1.5	0	5
Life satisfaction	3.8	1.2	1	5
Resilience	4.2	1.0	2	5
Optimism	3.5	1.1	1	5
Gratitude	3.2	1.0	1	5
Forgiveness	3.0	1.0	1	5
Empathy	3.8	1.1	1	5
Compassion	3.5	1.0	1	5
Kindness	3.2	1.0	1	5
Generosity	3.0	1.0	1	5
Patience	3.5	1.1	1	5
Self-control	3.8	1.0	1	5
Emotional stability	3.2	1.0	1	5
Psychological well-being	3.5	1.1	1	5
Life purpose	3.8	1.0	1	5
Meaning in life	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Humanistic well-being	3.5	1.1	1	5
Existential well-being	3.2	1.0	1	5
Transcendental well-being	3.0	1.0	1	5
Human				

(2) INFORMATION FOR SEQ ID NO:6:

(A) LENGTH: 255 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Asn Val Asp Ala Gly Leu Pro Arg Asn Ala Val Leu Tyr Ile Met Gly
 65 70 75 80
 Tyr Arg Ala Gly Asp Thr Ser Tyr Phe Phe Asn Glu Ala Ser Ala Thr
 85 90 95
 Glu Ala Ala Lys Tyr Val Phe Lys Asp Ala Met Arg Lys Val Thr Leu
 100 105 110
 Pro Tyr Ser Gly Asn Tyr Glu Arg Leu Gln Thr Ala Ala Gly Gly Leu
 115 120 125
 Arg Glu Asn Ile Pro Leu Gly Leu Pro Ala Leu Asp Ser Ala Ile Thr
 130 135 140
 Thr Leu Phe Tyr Tyr Asn Ala Asn Ser Ala Ala Ser Ala Leu Met Val
 145 150 155 160
 Leu Ile Gln Ser Thr Ser Glu Ala Ala Arg Tyr Lys Phe Ile Glu Gln
 165 170 175
 Gln Ile Gly Ser Arg Val Asp Lys Thr Phe Leu Pro Ser Leu Ala Ile
 180 185 190
 Ile Ser Leu Glu Asn Ser Leu Trp Leu Ala Leu Ser Lys Gln Ile Gln
 195 200 205
 Ile Ala Ser Thr Asn Asn Gly Glu Phe Glu Thr Pro Val Val Leu Ile
 210 215 220
 Asn Ala Gln Asn Gln Arg Val Thr Ile Thr Asn Val Asp Ala Gly Val
 225 230 235 240
 Val Thr Ser Asn Ile Ala Leu Leu Leu Asn Arg Asn Asn Met Ala
 245 250 255

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 263 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp Val Ser Phe Arg Leu Ser Gly Ala Asp Pro Arg Ser Tyr Gly Met
 1 5 10 15
 Phe Ile Lys Asp Leu Arg Asn Ala Leu Pro Phe Arg Glu Lys Val Tyr
 20 25 30
 Asn Ile Pro Leu Leu Leu Pro Ser Val Ser Gly Ala Gly Arg Tyr Leu
 35 40 45
 Leu Met His Leu Phe Asn Tyr Asp Gly Lys Thr Ile Thr Val Ala Val
 50 55 60
 Asp Val Thr Asn Val Tyr Ile Met Gly Tyr Leu Ala Asp Thr Thr Ser
 65 70 75 80
 Tyr Phe Phe Asn Glu Pro Ala Ala Glu Leu Ala Ser Gln Tyr Val Phe
 85 90 95

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0099270-25207960

1990-1991		1991-1992		1992-1993		1993-1994		1994-1995		1995-1996		1996-1997		1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217	
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(2) INFORMATION FOR SEQ ID NO:8:

(ii) MOLECULE TYPE: protein

Ala	Pro	Thr	Leu	Glu	Thr	Ile	Ala	Ser	Leu	Asp	Leu	Asn	Asn	Pro	Thr
1				5					10					15	
Thr	Tyr	Leu	Ser	Phe	Ile	Thr	Asn	Ile	Arg	Thr	Lys	Val	Ala	Asp	Lys
			20					25					30		
Thr	Glu	Gln	Cys	Thr	Ile	Gln	Lys	Ile	Ser	Lys	Thr	Phe	Thr	Gln	Arg
		35					40					45			
Tyr	Ser	Tyr	Ile	Asp	Leu	Ile	Val	Ser	Ser	Thr	Gln	Lys	Ile	Thr	Leu
	50					55					60				
Ala	Ile	Asp	Met	Ala	Asp	Leu	Tyr	Val	Leu	Gly	Tyr	Ser	Asp	Ile	Ala
65					70					75					80
Asn	Asn	Lys	Gly	Arg	Ala	Phe	Phe	Phe	Lys	Asp	Val	Thr	Glu	Ala	Val
				85					90					95	
Ala	Asn	Asn	Phe	Phe	Pro	Gly	Ala	Thr	Gly	Thr	Asn	Arg	Ile	Lys	Leu
			100					105					110		

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Variable	Mean	SD	Min	Max
Age	35.2	10.5	22	55
Gender	Male	10.2	0	20
Marital status	Married	15.8	0	30
Education	High school	12.5	0	25
Occupation	Manager	18.3	0	35
Income	\$30,000	15.0	0	60
Health	Good	10.0	0	20
Stress	Low	5.0	0	10
Life satisfaction	High	15.0	0	30
Work-life balance	Good	10.0	0	20
Family support	High	15.0	0	30
Community involvement	Low	5.0	0	10
Volunteer work	Low	5.0	0	10
Charitable contributions	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10
Family participation	Low	5.0	0	10
Community participation	Low	5.0	0	10
Volunteer participation	Low	5.0	0	10
Charitable participation	Low	5.0	0	10
Political participation	Low	5.0	0	10
Religious participation	Low	5.0	0	10
Cultural participation	Low	5.0	0	10
Environmental participation	Low	5.0	0	10
Health participation	Low	5.0	0	10
Education participation	Low	5.0	0	10
Work participation	Low	5.0	0	10

(2) INFORMATION FOR SEQ ID NO:9:

(ii) MOLECULE TYPE: protein

Ile	Asn	Thr	Ile	Thr	Phe	Asp	Ala	Gly	Asn	Ala	Thr	Ile	Asn	Lys	Tyr
1			5					10						15	
Ala	Thr	Phe	Met	Glu	Ser	Leu	Arg	Asn	Glu	Ala	Lys	Asp	Pro	Ser	Leu
			20					25					30		
Lys	Cys	Tyr	Gly	Ile	Pro	Met	Leu	Pro	Asn	Thr	Asn	Ser	Thr	Ile	Lys
		35					40					45			
Tyr	Leu	Leu	Val	Lys	Leu	Gln	Gly	Ala	Ser	Leu	Lys	Thr	Ile	Thr	Leu
	50					55					60				
Met	Leu	Arg	Arg	Asn	Asn	Leu	Tyr	Val	Met	Gly	Tyr	Ser	Asp	Pro	Tyr
65					70					75					80
Asp	Asn	Lys	Cys	Arg	Tyr	His	Ile	Phe	Asn	Asp	Ile	Lys	Gly	Thr	Glu
				85					90					95	
Tyr	Ser	Asp	Val	Glu	Asn	Thr	Leu	Cys	Pro	Ser	Ser	Asn	Pro	Arg	Val
			100					105					110		
Ala	Lys	Pro	Ile	Asn	Tyr	Asn	Gly	Leu	Tyr	Pro	Thr	Leu	Glu	Lys	Lys
		115					120					125			
Ala	Gly	Val	Thr	Ser	Arg	Asn	Glu	Val	Gln	Leu	Gly	Ile	Gln	Ile	Leu
	130					135					140				

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[illegible]

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 259 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val	Thr	Ser	Ile	Thr	Leu	Asp	Leu	Val	Asn	Pro	Thr	Ala	Gly	Gln	Tyr
1				5					10					15	
Ser	Ser	Phe	Val	Asp	Lys	Ile	Arg	Asn	Asn	Val	Lys	Asp	Pro	Asn	Leu
			20					25					30		
Lys	Tyr	Gly	Gly	Thr	Asp	Ile	Ala	Val	Ile	Gly	Pro	Pro	Ser	Lys	Glu
		35					40					45			
Lys	Phe	Leu	Arg	Ile	Asn	Phe	Gln	Ser	Ser	Arg	Gly	Thr	Val	Ser	Leu
	50					55					60				
Gly	Leu	Lys	Arg	Asp	Asn	Leu	Tyr	Val	Val	Ala	Tyr	Leu	Ala	Met	Asp
65					70					75					80
Asn	Thr	Asn	Val	Asn	Arg	Ala	Tyr	Tyr	Phe	Arg	Ser	Glu	Ile	Thr	Ser
				85					90					95	
Ala	Glu	Ser	Thr	Ala	Leu	Phe	Pro	Glu	Ala	Thr	Thr	Ala	Asn	Gln	Lys
			100					105					110		
Ala	Leu	Glu	Tyr	Thr	Glu	Asp	Tyr	Gln	Ser	Ile	Glu	Lys	Asn	Ala	Gln
		115					120					125			
Ile	Thr	Gln	Gly	Asp	Gln	Ser	Arg	Lys	Glu	Leu	Gly	Leu	Gly	Ile	Asp
		130				135					140				
Leu	Leu	Ser	Thr	Ser	Met	Glu	Ala	Val	Asn	Lys	Lys	Ala	Arg	Val	Val
145					150					155					160

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[illegible]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 813 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGCTAGATA	COGTGTCATT	CTCAACCAA	GGTGCCACTT	ATATTACCTA	CGTGAATTC	60
TTGAATGAGC	TACGAGTTAA	ATTGAAACCC	GAAGGTAACA	GCCATGGAAT	CCCATTGCTG	120
CGCAAAAAAT	GTGATGATCC	TGGAAAGTGT	TTCGTTTTGG	TAGCGCTTTC	AAATGACAA	180
GGACAGTTGG	CGGAAATAGC	TATAGATGTT	ACAAGTGTTT	ATGTGGTGGG	CTATCAAGTA	240
AGAAACAGAT	CTTACTTCTT	TAAAGATGCT	CCAGATGCTG	CTTACGAAGG	CCTCTTCAA	300
AACACAATTA	AAACAAGACT	TCATTTTGGC	GGCAGGTATC	CCTCGCTGGA	AGGTGAGAAG	360
GCATATAGAG	AGACAACAGA	CTTGGGCATT	GAACCATTAA	GGATTGGCAT	CAAGAAACTT	420
GATGAAAATG	CGATAGACAA	TTATAAACCA	ACGGAGATAG	CTAGTTCTCT	ATTGGTTGTT	480
ATTCAAATGG	TGTCTGAAGC	AGCTCGATT	ACCTTTATTG	AGAACCAAAT	TAGAAATAAC	540
TTTCAACAGA	GAATTCGCCC	GGCGAATAAT	ACAATCAGCC	TTGAGAATAA	ATGGGGTAAA	600
CTCTCGTTCC	AGATCCGGAC	ATCAGGTGCA	AATGGAATGT	TTTCGGAGGC	AGTTGAATTG	660
GAACGTGCAA	ATGGCAAAAA	ATACTATGTC	ACCGCAGTTG	ATCAAGTAAA	ACCCAAAAATA	720
GCACTCTTGA	AGTTCGTGCA	TAAAGATCCT	AAAACGAGCC	TTGCTGCTGA	ATTGATAATC	780
CAGAACTATG	AGTCATTAGT	GGGCTTTGAT	TAG			813

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(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 846 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

ATGGCGGGCAA AGATGGCGAA GAACGTGGAC AAGCGGCTCT TCACCGCGAC GTTCAACGTC      60
CAGGCCAGCT CCGCCGACTA CGCCACCTTC ATCGCCGGCA TCCGCAACAA GCTCCGCAAC      120
CCGGCGCACT TCTCCACAA CCGCCCCGTG CTGCGGCCGG TCGAGCCCAA CGTCCCCCGG      180
AGCAGGTGGT TCCACGTCGT GCTCAAGGCC TCGCCGACCA GCGCCGGGCT CACGCTGGCC      240
ATCCGCGCGG ACAACATCTA CCTGGAGGGC TTCAAGAGCA GCGACGGCAC CTGGTGGGAG      300
CTCACCCCGG GCCTCATCCC CGGCGCCACC TACGTGGGT TCGGCGGCAC CTACCGCGAC      360
CTCCTCGGCG ACACCGACAA GCTAACCAAC GTCGCTCTCG GCCGACAGCA GCTGGCGGAC      420
GCGGTGACCG CGTCCACGG GCGCACCAAG GCCGACAAGG CCTCCGGCCC GAAGCAGCAG      480
CAGGCGAGGG AGGCGGTGAC GACGCTGGTC CTCATGGTGA ACGAGGCCAC GCGGTTCAG      540
ACGGTGCTCG GGTTCGTGGC CGGGTTGCTG CACCCCAAGG CGGTGGAGAA GAAGAGCGGG      600
AAGATCGGCA ATGAGATGAA GGCCAGGTG AACGGGTGGC AGGACCTGTC CGCGGCGCTG      660
CTGAAGACGG ACGTGAAGCC TCCGCCGGA AAGTCGCCAG CGAAGTTCGC GCCGATCGAG      720
AAGATGGGCG TGAGGACGGC TGAACAGGCC GCCAACACGC TGGGGATCCT GCTGTTCTG      780
GAGGTGCCGG GTGGGTTGAC GGTGGCCAAG GCGCTGGAGC TGTTCATGC GAGTGGTGGG      840
AAATAG                                         846

```

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 913 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

CGTCCGAAAA TGGTGAAATG CTTACTACTT TCTTTTTTAA TTATCGCCAT CTTCAATTGGT      60
GTTCTACTG CCAAAGGCGA TGTTAACTTC GATTGTGCGA CTGCCACTGC AAAAACCTAC      120
ACAAAATTTA TCGAAGATT CAGGGCGACT CTTCCATTTA GCCATAAAGT GTATGATATA      180
CCTCTACTGT ATTCCACTAT TTCCGACTCC AGACGTTTCA TACTCCTCGA TCTTACAAGT      240
TATGCATATG AAACCATCTC GGTGGCCATA GATGTGACGA ACGTTTATGT TGTGGCGTAT      300

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09610030-076410

1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23		24		25		26		27		28		29		30		31		32		33		34		35		36		37		38		39		40		41		42		43		44		45		46		47		48		49		50		51		52		53		54		55		56		57		58		59		60		61		62		63		64		65		66		67		68		69		70		71		72		73		74		75		76		77		78		79		80		81		82		83		84		85		86		87		88		89		90		91		92		93		94		95		96		97		98		99		100	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100																																																																																																				

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGNYTNGAYA CNGTNWSNTT YWSNACNAAR GG

32

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATGGTTCAA TGCCCAAGTC TGT

23

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGTCTCTCTA TATGCCTTTC CAC

23

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCAACCCGGG CTAGATACCG TGTCAATTCTC AACCAAAGGT GCCACTTATA TTA

53

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTTCATTTTG GCGGCACGTA TCC

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(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTCGAGGCTG CAAGCTTACG TGGGATTTT TTTTTTTT TTTTTT

46

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTCGCTGGAA GGTGAGAA

18

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCGAGGCTG CAAGCTTACG TGGGA

25

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGATCTCGAG TACTATTAG GATCTTATC GACGA

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(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTAAGCAGCA TCTGGAGCAT CT

22

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CATTCAAGAA ATTACGCTAG G

21

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGCCTGGACA CCGTGAGCTT TAG

23

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TCGATTGCGA TCCTAAATAG TACTC

25

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(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTAGGATCG CAATCGACGA ACTTCAAG

28

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTTTCGTCTGT AAAGATCCTA AATAGTACTC GA

32

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGATCTTTAC AGACGAACTT CAAGAGT

27

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCTTGTGCTT CGTCGATAAA GATCC

25

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(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATCGACGAAG CACAAGAGTG CTATTTT

27

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTAAAACCAT GCATAGCACT CTTGAAGTTC GT

32

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AGTGCTATGC ATGGTTTAC TTGATCAACT GC

32

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGCACATGTG GTGCCACTTA TATTACCTA

29

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(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TAAGTGGCAC CACATGTGCT AAAGCTCAGC GTG

33

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TGACTGTGGA CAGTTGGCGG AAATA

25

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCCACTGTCC ACAGTCATTT GAAAGCGCTA CC

32

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GATGATCCTG GAAAGGCTTT CGTTTTGGTA GCGCTT

36

093109130 070600

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(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAGCCTTTCC AGGATCATCA GCTTTTTTGG GCAGCAATGG G

41

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AAGCCTTTCC AGGATCATCA CAT

23

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCGACTCTCT ACTGTTTC

18

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGTTAGCAAT TTAAGTGTGA T

21

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(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AACAGCTATG ACCATG

16

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TGAACCTCGAG GAACTACCT ATTTCCCAC

29

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCATTACATC CATGGCGGC

19

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 64 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GATATCTCGA GTTAATATT TCCACCACA CGCATGGAAC AGCTCCAGCG CTTGGCCAC

60

CGTC

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1990-1991		1991-1992		1992-1993		1993-1994		1994-1995		1995-1996		1996-1997		1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217	
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

21

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

21

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

27

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

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(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

ATTGGNAGDG TAGCCCTRAA RTCYTCDAT

29

(2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GCCACTGCAA AAACCTACAC AAAATTTATT GA

32

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GATGTAACT TCGATTGTC GA

22

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TCAACTCGAG GTACTCAATT CACAACAGAT TCC

33

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(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 813 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

```

GGCCTGGACA CCGTGAGCTT TAGCACTAAA GGTGCCACTT ATATTACCTA CGTGAATTC      60
TTGAATGAGC TACGAGTTAA ATTGAAACCC GAAGGTAACA GCCATGGAAT CCCATTGCTG      120
CGCAAAAAAT GTGATGATCC TGGAAAGTGT TTCGTTTGGG TAGCGCTTTC AAATGACAAT      180
GGACAGTTGG CGGAAATAGC TATAGATGTT ACAAGTGTTT ATGTGGTGGG CTATCAAGTA      240
AGAAACAGAT CTTACTTCTT TAAAGATGCT CCAGATGCTG CTTACGAAGG CCTCTTCAAA      300
AACACAATTA AAACAAGACT TCATTTTGGC GGCACGTATC CCTCGCTGGA AGGTGAGAAG      360
GCATATAGAG AGACAACAGA CTTGGGCATT GAACCATTAA GGATTGGCAT CAAGAAACTT      420
GATGAAAATG CGATAGACAA TTATAAACCA ACGGAGATAG CTAGTTCTCT ATTGGTTGTT      480
ATTCAAATGG TGCTGAAGC AGCTCGATTC ACCTTTATTG AGAACCAAAT TAGAAATAAC      540
TTTCAACAGA GAATTCGCCC GCGGAATAAT ACAATCAGCC TTGAGAATAA ATGGGGTAAA      600
CTCTCGTTCC AGATCCGGAC ATCAGGTGCA AATGGAATGT TTTCCGAGGC AGTTGAATTG      660
GAACGTGCAA ATGGCAAAAA ATACTATGTC ACCGCAGTTG ATCAAGTAAA ACCCAAATA      720
GCACTCTTGA AGTTCGTGCA TAAAGATCCT AAAACGAGCC TTGCTGCTGA ATTGATAATC      780
CAGAACTATG AGTCATTAGT GGGCTTTGAT TAG                                     813

```

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

```

Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
 1           5           10           15
Pro Ser Met Cys
                20

```

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Pro Ser Gly Gln Ala Gly Ala Ala Ala Ser Glu Ser Leu Phe Ile Ser
 1 5 10 15
 Asn His Ala Tyr
 20

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CAGCCATGGA ATCCCATTCG TG

22

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CACATGTAAA ACAAGACTTC ATTTTGGC

28

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

TGAAGTCTTG TTTTAGATGT GTTTTGAAG AGGCCT

36

(2) INFORMATION FOR SEQ ID NO:63:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

ATGCCATATG CAATTATAAA CCAACGGAGA

30

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GGTTTATAAT TGCATATGGC ATTTTCATCA AGTTTCTTG

39

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CTTTCAACAA TGCATTCGCC CGGCGAATAA TAC

33

(2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GCGAATGCAT TGTGAAAGT TATTTCTAAT TTG

33

(2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GTTTTGTGAG GCAGTTGAAT TGGAAC

26

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TTCAACTGCC TCACAAAACA TTCCATTTC ACCT

34

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

AAAAGCTGAT GATCCTGGAA AGTG

24

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

TCCAGGATCA TCAGCTTTTT TGGCAGCAA TGGGA

35

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 321 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GACATCCAGA TGACTCAGTC TCCATCTTCC ATGTCTGCAT CTCTGGGAGA CAGAGTCACT

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[illegible]

(2) INFORMATION FOR SEQ ID NO:72:

(ii) MOLECULE TYPE: DNA

CAGATCCAGT	TGGTGCAGTC	TGGACCTGGC	CTGAAGAAGC	CTGGAGGGTC	CGTCAGAATC	60
TCCTGCGCAG	CTTCTGGGTA	TACCTTCACA	AACTATGGAA	TGAACTGGGT	GAAGCAGGCT	120
CCAGGAAAGG	GTTTAAGGTG	GATGGGCTGG	ATAAACACCC	ACACTGGAGA	GCCAACATAT	180
GCTGATGACT	TCAAGGGACG	GTTTACCTTC	TCTTTGGACA	CGTCTAAGAG	CACTGCCTAT	240
TTACAGATCA	ACAGCCTCAG	AGCCGAGGAC	ACGGCTACAT	ATTTCTGTAC	AAGACGGGGT	300
TACGACTGGT	ACTTCGATGT	CTGGGGCCAA	GGGACCACGG	TCACCGTCTC	CTCC	354

(2) INFORMATION FOR SEQ ID NO:73:

(ii) MOLECULE TYPE: DNA

GAGATCCAGT	TGGTGCAGTC	TGGAGGAGGC	CTGGTGAAGC	CTGGAGGGTC	CGTCAGAATC	60
TCCTGCGCAG	CTTCTGGGTA	TACCTTCACA	AACTATGGAA	TGAACTGGGT	GCGCCAGGCT	120
CCAGGAAAGG	GTTTAGAGTG	GATGGGCTGG	ATAAACACCC	ACACTGGAGA	GCCAACATAT	180
GCTGATTCTT	TCAAGGGACG	GTTTACCTTC	TCTTTGGACG	ATTCTAAGAA	CACTGCCTAT	240
TTACAGATCA	ACAGCCTCAG	AGCCGAGGAC	ACGGCTGTGT	ATTTCTGTAC	AAGACGGGGT	300
TACGACTGGT	ACTTCGATGT	CTGGGGCCAA	GGGACCACGG	TCACCCTCTC	CTCC	354

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(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 321 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

```

GACATCCAGA TGACTCAGTC TCCATCTTCC CTGTCTGCAT CTGTAGGAGA CAGAGTCACT      60
ATCACTTGCC GGGCGAGTCA GGACATTAAT AGCTATTTAA GCTGGTTCCA GCAGAAACCA      120
GGGAAAGTCT CTAAGACCCT GATCTATCGT GCAAACAGAT TGGAACTCTGG GGTCCCATCA      180
AGGTTTCAGTG GCAGTGGATC TGGGACAGAT TATACTCTCA CCATCAGCAG CCTGCAATAT      240
GAAGATTTTG GAATTTATTA TTGTCAACAG TATGATGAGT CTCCTGGAC GTTCGGTGGG      300
GGCACCAAGC TTGAAATCAA A                                          321

```

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

```

TGTCATCATC ATGCATCGCG AGTTGCCAGA ATGGCATCTG ATGAGTTTCC TTCTATGTGC      60
GCAAGTACTC                                          70

```

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

```

TCGAGAGTAC TTGCCACAT AGAAGGAAAC TCATCAGATG CCATTCTGGC AACTCGCGAT      60
GCATGATGAT GACATGCA                                          78

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(2) INFORMATION FOR SEQ ID NO:77:

- (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

30

(2) INFORMATION FOR SEQ ID NO:78:

- (11) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

15

(2) INFORMATION FOR SEQ ID NO:79:

- (ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

18

(2) INFORMATION FOR SEQ ID NO:80:

- (ii) MOLECULE TYPE: DNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:80:

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(2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 76 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CTAACTCGAG AGTACTGTAT GCATGGTTCG AGATGAACAA AGATTCTGAG GCTGCAGCTC 60
CAGCCTGTCC AGATGG 76

(2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CTAACTCGAG AGTACTGTAT 20

(2) INFORMATION FOR SEQ ID NO:83:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

TCCAGCCTGT CCAGATGGAC ACTCTCCCCT GTTGAA 36

(2) INFORMATION FOR SEQ ID NO:84:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

GTACAGTCCA AGGTGGAT 18

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(2) INFORMATION FOR SEQ ID NO:85:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

CATGCCGCCG ATTAGGATC TTTATCGACG A

31

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

AACATCCAGT TCGTGCACTC TG

22

(2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

GAGGAGACGG TGACCGTGGT

20

(2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

GACATCAAGA TGACCCAGT

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(2) INFORMATION FOR SEQ ID NO:89:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

GTTTGATTTC AAGCTTGGTG C

21

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

ACTTCGGCCG CACCATCTGG ACAGGCTGGA G

31

(2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CGTTAGCAAT TTAAGTGTGA T

21

(2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 723 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

GACATCCAGA TGAAGCAGTC TCCATCTTCC CTGTCTGCAT CTGTAGGAGA CAGAGTCACT

60

ATCACTTGCC GGGCGAGTCA GGACATTAAT AGCTATTTAA GCTGGTTCCA GCAGAAACCA

120

GGGAAAGCTC CTAAGACCCT GATCTATCGT GCAAACAGAT TGAATCTGG GGTCCCATCA

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AGGTTTCAGTG GCAGTGGATC TGGGACAGAT TATACTCTCA CCATCAGCAG CCTGCAATAT 240
 GAAGATTTTG GAATTTATTA TTGTCACAG TATGATGAGT CTCCGTGGAC GTTCGGTGGA 300
 GGCACCAAGC TTGAGATGAA AGGTGGCGGT GGATCTGGTG GAGGTGGGTC CGGAGGTGGA 360
 GGATCTGAGA TCCAGTTGGT GCAGTCTGGA GGAGGCCTGG TGAAGCCTGG AGGGTCCGTC 420
 AGAATCTCCT GCGCAGCTTC TGGGTATACC TTCACAACT ATGGAATGAA CTGGGTGCGC 480
 CAGGCTCCAG GAAAGGGTTT AGAGTGGATG GGCTGGATAA ACACCCACAC TGGAGAGCCA 540
 ACATATGCTG ATTCTTTCAA GGGACGGTTT ACCTTCTCTT TGGACGATTC TAAGAACACT 600
 GCCTATTAC AGATCAACAG CCTCAGTCC GAGGACACGG CTGTGTATT CTGTACAAGA 660
 CGGGGTTACG ACTGGTACTT CGATGTCTGG GGCCAAGGGA CCACGGTCAC CGTCTCCTCA 720
 TGA 723

(2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 723 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GAGATCCAGT TGGTGCACTC TGGAGGAGGC CTGGTGAAGC CTGGAGGGTC CGTCAGAATC 60
 TCCTGCGCAG CTTCTGGGTA TACCTTCACA AACTATGGAA TGAAGTGGT GCGCCAGGCT 120
 CCAGGAAAGG GTTAGAGTG GATGGGCTGG ATAAACACCC AACTGGAGA GCCAACATAT 180
 GCTGATTCTT TCAAGGGACG GTTTACCTTC TCTTTGGACG ATTCTAAGAA CACTGCCTAT 240
 TTACAGATCA ACAGCCTCAG AGCCGAGGAC ACGGCTGTGT ATTTCTGTAC AAGACGGGGT 300
 TACGACTGGT ACTTCGATGT CTGGGGCCAA GGGACCACGG TCACCGTCTC CTCAGGTGGC 360
 GGTGGATCTG GTGGAGGTGG GTCCGGAGGT GGAGGATCTG ACATCCAGAT GACTCAGTCT 420
 CCATCTTCCC TGTCTGCATC TGTAGGAGAC AGAGTCACTA TCACTTGCCG GCGGAGTCAG 480
 GACATTAATA GCTATTTAAG CTGGTTCCAG CAGAAACCAG GGAAGCTCC TAAGACCCTG 540
 ATCTATCGTG CAAACAGATT GGAATCTGGG GTCCCATCAA GGTTCAGTGG CAGTGGATCT 600
 GGGACAGATT ATACTCTCAC CATCAGCAGC CTGCAATATG AAGATTTTGG AATTTATTAT 660
 TGTCAACAGT ATGATGAGTC TCCGTGGACG TTCGGTGGAG GCACCAAGCT TGAGATGAAA 720
 TGA 723

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 51 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

CGGACCCACC TCCACCAGAT CCACCGCCAC CTTTCATCTC AAGCTTGGTG C

51

(2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GACATCCAGA TGACTCAGT

19

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 49 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GGTGGAGGTG GGTCCGGAGG TCGAGGATCT GAGATCCAGT TGGTGCCAGT

49

(2) INFORMATION FOR SEQ ID NO:97:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

TGTACTCGAG CCCATCATGA GGAGACGGTG ACCGT

35

(2) INFORMATION FOR SEQ ID NO:98:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:
GGTGGAGGTG GGTCCGGAGG TGGAGGATCT 30
- (2) INFORMATION FOR SEQ ID NO:99:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:
TGTACTCGAG CCCATCATT CATCTCAAGC TTGGTGC 37
- (2) INFORMATION FOR SEQ ID NO:100:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:
GAGATCCAGT TGGTGCAGTC TG 22
- (2) INFORMATION FOR SEQ ID NO:101:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:
CGGACCCACC TCCACCAGAT CCACCGCCAC CTGAGGAGAC GGTGACCGT 49

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the <u>microorganisms</u> referred to in the description on page <u>12</u> lines <u>11-27</u>	
B. IDENTIFICATION OF DEPOSIT Deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit See attached sheet	Accession Number See attached sheets
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
"In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 23(4) EPC)." 	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EPO	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer <i>Kevin R. Miller</i>	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer

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CLAIMS

1. An analog of a Type I ribosome-inactivating protein, said analog having a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.
2. The analog recited in claim 1 wherein said Type I ribosome-inactivating protein is gelonin.
3. The analog recited in claim 2 wherein said cysteine is at a position in said analog from position 244 to the carboxyl terminal position of said analog.
4. The analog recited in claim 3 wherein said cysteine is at a position in said analog from position 247 to the carboxyl terminal position of said analog.
5. The analog recited in claim 3 wherein said cysteine is at position 244 of the amino acid sequence of said analog.
6. The analog recited in claim 4 wherein said cysteine is at position 247 of the amino acid sequence of said analog.
7. The analog recited in claim 2 wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues.
8. The analog recited in claim 4 wherein said cysteine is at position 248 of the amino acid sequence of said analog.
9. The analog recited in claim 1 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

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10. The analog recited in claim 9 wherein said cysteine is at a position in said analog from position 256 to the carboxyl terminal position of said analog.

11. The analog recited in claim 10 wherein said cysteine is at a position in said analog from position 260 to the carboxyl terminal position of said analog.

12. The analog recited in claim 10 wherein said cysteine is at position 256 of the amino acid sequence of said analog.

13. The analog recited in claim 11 wherein said cysteine is at position 270 of the amino acid sequence of said analog.

14. The analog recited in claim 11 wherein said cysteine is at position 277 of the amino acid sequence of said analog.

15. The analog recited in claim 1 wherein said Type I ribosome-inactivating protein is momordin II.

16. The analog recited in claim 1 wherein said position of said cysteine in the amino acid sequence of said analog corresponds to a position within one amino acid of position 259 of SEQ ID NO: 1.

17. The analog recited in claim 16 wherein said Type I ribosome-inactivating protein is gelonin.

18. The analog recited in claim 16 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

19. The analog recited in claim 16 wherein said Type I ribosome-inactivating protein is momordin II.

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20. The analog recited in claim 1 wherein said position of said cysteine in the amino acid sequence of said analog corresponds to position 259 of SEQ ID NO: 1.

5 21. The analog recited in claim 20 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

22. The analog recited in claim 20 wherein said Type I ribosome-inactivating protein is momordin II.

10 23. A polynucleotide encoding an analog of a Type I ribosome-inactivating protein, said analog having a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

15 24. The polynucleotide recited in claim 23 wherein said Type I ribosome-inactivating protein is gelonin.

25. The polynucleotide recited in claim 24 wherein said cysteine is at a position in said analog from position 244 to the carboxyl terminal position of said analog.

20 26. The polynucleotide recited in claim 25 wherein said cysteine is at a position in said analog from position 247 to the carboxyl terminal position of said analog.

27. The polynucleotide recited in claim 25 wherein said cysteine is at position 244 of the amino acid sequence of said analog.

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28. The polynucleotide recited in claim 26 wherein said cysteine is at position 247 of the amino acid sequence of said analog.

29. The polynucleotide recited in claim 24 wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues.

5 30. The polynucleotide recited in claim 26 wherein said cysteine is at position 248 of the amino acid sequence of said analog.

31. The polynucleotide recited in claim 23 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

10 32. The polynucleotide recited in claim 31 wherein said cysteine is at a position in said analog from position 256 to the carboxyl terminal position of said analog.

33. The analog recited in claim 32 wherein said cysteine is at a position in said analog from position 260 to the carboxyl terminal position of said analog.

15 34. The polynucleotide recited in claim 32 wherein said cysteine is at position 256 of the amino acid sequence of said analog.

35. The polynucleotide recited in claim 33 wherein said cysteine is at position 270 of the amino acid sequence of said analog.

20 36. The polynucleotide recited in claim 33 wherein said cysteine is at position 277 of the amino acid sequence of said analog.

37. The polynucleotide recited in claim 23 wherein said Type I ribosome-inactivating protein is mormordin II.

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38. A vector encoding an analog of a Type I ribosome-inactivating protein, said analog having a cysteine available for intermolecular disulfide bonding at a amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

39. A host cell comprising a DNA vector including a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, said analog having a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

40. The host cell recited in claim 39 wherein said Type I ribosome-inactivating protein is gelonin.

41. The host cell recited in claim 40 wherein said cysteine is at position 247 of the amino acid sequence of said analog.

42. The host cell recited in claim 41 wherein said host cell is of the type deposited as ATCC Accession No. 69009.

43. The host cell recited in claim 39 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

44. The host cell recited in claim 43 wherein said cysteine is at position 277 of the amino acid sequence of said analog.

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45. The host cell recited in claim 44 wherein said host cell is of the type deposited as ATCC Accession No. 68722.

46. An agent toxic to a cell comprising an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to said cell, said cysteine being located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

47. The agent recited in claim 46 wherein said Type I ribosome-inactivating protein is gelonin.

48. The agent recited in claim 47 wherein said cysteine is at a position in said analog from position 247 to the carboxyl terminal position of said analog.

49. The agent recited in claim 48 wherein said cysteine is at position 247 of the amino acid sequence of said analog.

50. The agent recited in claim 47 wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues.

51. The agent recited in claim 48 wherein said cysteine is at position 248 of the amino acid sequence of said analog.

52. The agent recited in claim 46 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

53. The agent recited in claim 52 wherein said cysteine is at a position in said analog from position 260 to the carboxyl terminal position of said analog.

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54. The agent recited in claim 53 wherein said cysteine is at a position in said analog from position 270 to the carboxyl terminal position of said analog.

55. The agent recited in claim 46 wherein said cysteine is at position 256 of the amino acid sequence of said analog.

5 56. The agent recited in claim 54 wherein said cysteine is at position 270 of the amino acid sequence of said analog.

57. The agent recited in claim 54 wherein said cysteine is at position 277 of the amino acid sequence of said analog.

10 58. The agent recited in claim 46 wherein said Type I ribosome-inactivating protein is momordin II.

59. The agent recited in claim 47 wherein said Type I ribosome-inactivating protein is linked to an antibody.

60. The agent recited in claim 59 wherein said Type I ribosome-inactivating protein is linked to an H65 antibody.

15 61. The agent recited in claim 47 wherein said Type I ribosome-inactivating protein is linked to an antibody fragment.

62. The agent recited in claim 61 wherein said Type I ribosome-inactivating protein is linked to an antibody fragment selected from the group consisting of chimeric and human engineered antibody fragments.

20 63. The agent recited in claim 61 wherein said antibody fragment is selected from the group consisting of a Fab antibody fragment, a Fab' antibody fragment and a F(ab')₂ antibody fragment.

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64. The agent recited in claim 62 wherein said antibody fragment is selected from the group consisting of a Fab antibody fragment, a Fab' antibody fragment and a F(ab')₂ antibody fragment.

5 65. A method for preparing an analog of a Type I ribosome-inactivating protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein, said cysteine being located at
10 a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

15 66. The product of a method for preparing an analog of a Type I ribosome-inactivating protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein, said cysteine being located at
a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

20 67. A method for preparing an agent toxic to a cell comprising the step of linking an analog of a Type I ribosome-inactivating protein through a cysteine to a molecule which specifically binds to said cell, said analog having said cysteine at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said
25 cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

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68. A method for treating a disease in which elimination of particular cells is a goal, comprising the step of administering to a patient having said disease a therapeutically effective amount of an agent toxic to said cells comprising an analog of a Type I ribosome-inactivating protein linked through a cysteine to a molecule which specifically binds to said cell, said analog having said cysteine at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and said cysteine being located at a position in the amino acid sequence of said analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said analog.

69. An analog of a Type I ribosome-inactivating protein, wherein said analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein said analog retains ribosome-inactivating activity of said Type I ribosome-inactivating protein.

70. The analog recited in claim 69 wherein said Type I ribosome inactivating protein is gelonin.

71. The analog recited in claim 70 wherein said cysteine is at position 10 of the amino acid sequence of said analog.

72. A host cell comprising a vector encoding an analog according to claim 71, wherein said host cell is of the type deposited as ATCC Accession No. 69008.

73. The analog recited in claim 70 wherein said cysteine is at a position 60 in the amino acid sequence of said analog.

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74. The analog recited in claim 69 wherein said Type I ribosome-inactivating protein includes a single cysteine.

75. The analog recited in claim 74 wherein said Type I ribosome-inactivating protein is gelonin.

5 76. The analog recited in claim 75 wherein said cysteine is at position 10 in the amino acid sequence of said analog.

77. The analog recited in claim 75 wherein said cysteine is at position 44 in the amino acid sequence of said analog.

10 78. The analog recited in claim 75 wherein said cysteine is at position 50 in the amino acid sequence of said analog.

79. The analog recited in claim 75 wherein said cysteine is at position 247 in the amino acid sequence of said analog.

15 80. A polynucleotide encoding an analog of a Type I ribosome-inactivating protein, wherein said analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in said Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein said analog retains ribosome-inactivating activity of said Type I ribosome-inactivating protein.

20

81. A method for preparing an analog of a Type I ribosome-inactivating protein comprising the step of expressing in suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position
25 corresponding to a position not naturally available for disulfide bonding in said Type

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I ribosome-inactivating protein, said cysteine being located at a position corresponding to an amino acid position on the surface of ricin A-chain in its natural conformation and said analog retaining ribosome-inactivating activity of said Type I ribosome-inactivating protein.

5 82. An agent toxic to a cell comprising an analog of a Type I
ribosome-inactivating protein linked by a disulfide bond through a cysteine to a
molecule which specifically binds to said cell, wherein said analog has a cysteine
available for intermolecular disulfide bonding located at an amino acid position
10 corresponding to a position not naturally available for intermolecular disulfide
bonding in said Type I ribosome-inactivating protein and corresponding to a position
on the surface of ricin A-chain in its natural conformation, and wherein said analog
retains ribosome-inactivating activity of said Type I ribosome-inactivating protein.

15 83. A method for preparing an agent toxic to a cell comprising the
step of linking an analog of a Type I ribosome-inactivating protein through a cysteine
to a molecule which specifically binds to said cell, wherein said analog has a cysteine
available for intermolecular disulfide bonding located at an amino acid position
corresponding to a position not naturally available for intermolecular disulfide
20 bonding in said Type I ribosome-inactivating protein and corresponding to a position
on the surface of ricin A-chain in its natural conformation, and wherein said analog
retains ribosome-inactivating activity of said Type I ribosome-inactivating protein.

25 84. A method for treating a disease in which elimination of particular
cells is a goal, comprising the step of administering to a patient having said disease
a therapeutically effective amount of an agent toxic to said cells comprising an analog
of a Type I ribosome-inactivating protein linked by a disulfide bond through a
cysteine to a molecule which specifically binds to said cell, wherein said analog has
a cysteine available for intermolecular disulfide bonding located at an amino acid
position corresponding to a position not naturally available for intermolecular
disulfide bonding in said Type I ribosome-inactivating protein and corresponding to
a position on the surface of ricin A-chain in its natural conformation, and wherein

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said analog retains ribosome-inactivating activity of said Type I ribosome-inactivating protein.

85. A purified and isolated polynucleotide encoding natural sequence gelonin.

5 86. A host cell comprising a vector having an insert encoding gelonin wherein said host cell is of the type deposited as ATCC Accession No. 68721.

87. A purified and isolated polynucleotide encoding natural sequence barley ribosome-inactivating protein.

88. A purified and isolated polynucleotide encoding momordin II.

10 89. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin and a portion of an antibody having the capacity to specifically bind an antigen.

90. A polynucleotide encoding a fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin and a portion of
15 an antibody having the capacity to specifically bind an antigen.

91. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction of a portion of an antibody having the capacity to specifically bind an antigen. and gelonin.

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92. A polynucleotide encoding a fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, and gelonin.

5 93. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin, a peptide segment having an endopeptidase cleavage site and a portion of an antibody having the capacity to specifically bind an antigen.

10 94. A polynucleotide encoding a fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin, a peptide segment having an endopeptidase cleavage site and a portion of an antibody having the capacity to bind an antigen.

15 95. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, a peptide segment having an endopeptidase cleavage site, and gelonin.

96. A polynucleotide encoding a fusion protein coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, a peptide segment having an endopeptidase cleavage site, and gelonin.

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97. The fusion protein recited in claim 93 or 95 wherein said peptide segment is a segment of shiga-like toxin.

98. The fusion protein recited in claim 93 or 95 wherein said peptide segment is a segment of rabbit muscle aldolase.

5 99. The polynucleotide recited in claim 94 or 96 wherein said peptide segment is a segment of shiga-like toxin.

100. The polynucleotide recited in claims 94 or 96 wherein said peptide segment is a segment of rabbit muscle aldolase.

10 101. The fusion protein recited in claim 93 or 95 wherein said portion of said antibody is a kappa chain of a Fab fragment.

102. The polynucleotide recited in claim 94 or 96 wherein said portion of said antibody is a kappa chain of a Fab fragment.

103. The fusion protein recited in claim 93 or 95 wherein said portion of said antibody is an Fd chain of a Fab fragment.

15 104. The polynucleotide recited in claim 94 or 96 wherein said portion of said antibody is an Fd chain of a Fab fragment.

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105. The fusion protein recited in claim 93 or 95 wherein said portion of said antibody is a single chain antibody.

106. The polynucleotide recited in claim 94 or 96 wherein said portion of said antibody is a single chain antibody.

5 107. The fusion protein recited in claim 93 or 95 wherein said antibody is an H65 antibody.

108. The polynucleotide recited in claim 94 or 96 wherein said antibody is an H65 antibody.

10 109. The polynucleotide recited in claim 94 wherein said peptide segment is a segment of shiga-like toxin and said portion of said antibody is an Fd chain of a Fab fragment of an H65 antibody.

110. A vector comprising the polynucleotide recited in claim 109.

111. A host cell transformed or transfected with the vector recited in claim 110.

15 112. The host cell recited in claim 111 of the type deposited as ATCC 69102.

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113. The polynucleotide recited in claim 94 wherein said peptide segment is a segment of rabbit muscle aldolase and said portion of said antibody is a kappa chain of an H65 antibody.

114. A vector comprising the polynucleotide recited in claim 113.

5 115. A host cell transformed or transfected with the vector recited in claim 114.

116. The host cell recited in claim 115 of the type deposited as ATCC 69103.

10 117. The polynucleotide recited in claim 94 wherein said peptide segment is a segment of rabbit muscle aldolase and said portion of said antibody is an Fd chain of an H65 antibody.

118. A vector recited in claim 94 comprising the polynucleotide of claim 117.

15 119. A host cell transformed or transfected with the vector recited in claim 118.

120. The host cell recited in claim 120 of the type deposited as ATCC 69104.

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AMENDED CLAIMS

[received by the International Bureau on 6 April 1993 (06.04.93); ;
original claims 33, 96 and 120 amended; new claims 121-136 added;
remaining claims unchanged (6 pages)]

28. The polynucleotide recited in claim 26 wherein said cysteine is at position 247 of the amino acid sequence of said analog.

29. The polynucleotide recited in claim 24 wherein the native geonin cysteine residues at positions 44 and 50 are replaced with alanine residues.

30. The polynucleotide recited in claim 26 wherein said cysteine is at position 248 of the amino acid sequence of said analog.

31. The polynucleotide recited in claim 23 wherein said Type I ribosome-inactivating protein is barley ribosome-inactivating protein.

32. The polynucleotide recited in claim 31 wherein said cysteine is at a position in said analog from position 256 to the carboxyl terminal position of said analog.

33. The polynucleotide recited in claim 32 wherein said cysteine is at a position in said analog from position 260 to the carboxyl terminal position of said analog.

34. The polynucleotide recited in claim 32 wherein said cysteine is at position 256 of the amino acid sequence of said analog.

35. The polynucleotide recited in claim 33 wherein said cysteine is at position 270 of the amino acid sequence of said analog.

36. The polynucleotide recited in claim 33 wherein said cysteine is at position 277 of the amino acid sequence of said analog.

37. The polynucleotide recited in claim 23 wherein said Type I ribosome-inactivating protein is mormordin II.

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93. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin, a peptide segment having an endopeptidase cleavage site and a portion of an antibody having the capacity to specifically bind an antigen.

94. A polynucleotide encoding a fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for gelonin, a peptide segment having an endopeptidase cleavage site and a portion of an antibody having the capacity to bind an antigen.

95. A fusion protein comprising a sequence of amino acids coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, a peptide segment having an endopeptidase cleavage site, and gelonin.

96. A polynucleotide encoding a fusion protein coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, a peptide segment having an endopeptidase cleavage site, and gelonin.

114. A vector comprising the polynucleotide recited in claim 113.

116. The host cell recited in claim 115 of the type deposited as ATCC 69103.

118. A vector recited in claim 94 comprising the polynucleotide of claim 117.

119. A host cell transformed or transfected with the vector recited in claim 118.

120. The host cell recited in claim 119 of the type deposited as ATCC 69104.

121. The fusion protein recited in claim 89 or 91 wherein said portion of said antibody is a single chain antibody.

122. The fusion protein recited in claim 90 or 92 wherein said portion of said antibody is a single chain antibody.

123. A method for preparing a fusion protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a sequence of amino acids coding in the amino to carboxyl direction for gelonin and a portion of an antibody having the capacity to specifically bind an antigen.

124. A method for preparing a fusion protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a sequence of amino acids coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, and gelonin.

125. A method for preparing a fusion protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a sequence of amino acids coding in the amino to carboxyl direction for gelonin, a peptide segment having an endopeptidase cleavage site and a portion of an antibody having the capacity to specifically bind an antigen.

126. A method for preparing a fusion protein comprising the step of expressing in a suitable host cell a polynucleotide encoding a sequence of amino acids coding in the amino to carboxyl direction for a portion of an antibody having the capacity to specifically bind an antigen, a peptide segment having an endopeptidase cleavage site, and gelonin.

127. The analog recited in claim 7 wherein said cysteine available for disulfide bonding is position 247 of the amino acid sequence of said analog.

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128. The analog recited in claim 70 wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with alanine residues.

129. The analog recited in claim 128 wherein said cysteine residue available for disulfide bonding is at position 10 of the amino acid sequence of said analog.

130. The analog recited in claim 70 wherein said cysteine residue is at position 103 of the amino acid sequence of said analog.

131. The analog recited in claim 70 wherein said cysteine residue is at position 146 of the amino acid sequence of said analog.

132. The analog recited in claim 70 wherein said cysteine residue is at position 184 of the amino acid sequence of said analog.

133. The analog recited in claim 70 wherein said cysteine residue is at position 215 of the amino acid sequence of said analog.

134. A host cell comprising a vector encoding the analog recited in claim 78, wherein said host cell is of the type deposited as ATCC 69101.

135. A method for purifying a protein comprising a ribosome-inactivating protein and a portion of an antibody, said method comprising the steps of:
passing, through an anion exchange column, a solution containing a protein comprising a ribosome-inactivating protein and a portion of an antibody;
after said passing step, applying to a protein G column said protein; and
eluting said protein from said protein G column.

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136. The method as recited in claim 135 further comprising the steps of:

prior to said applying step, introducing the flow-through of the anion exchange column into a cation exchange column; and

then exposing the cation exchange column to an eluent effective to elute said protein.

RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLLTGADVRHEIPVLPNRVG	50
GELONIN	GLD----TVSFSTKGATYITYVNFNLNRVCLKPEGN-SHGIPLLRKKCD	45
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVGVRAGNSAYFFHPDNQED	100
GELONIN	DP-GKCFVLVALSNDNGQLAEIAIDVTSVYVVGVDVRNRSYFF----KDA	90
RTA	AEAITHLFTDVONRYTFAFGGNYDRLEQLAGNLRNIELGNGLPEEAISA	150
GELONIN	PDAAYEGLFKNTIKTRLHFHGGTYPSELEG-EKAYRETTDLGIEPLRIGIKK	139
RTA	LYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGEMRTRIRYNRRSAP	200
GELONIN	LDENAIIDNYKPTIEASSLLVVIOMVSEARFTFIENQIRNN--FQQRIRP	187
RTA	DPSVITLENSWGRSLTAIQESN-QGAFASPIQLQRRNGSKFSYVDVSILI	249
GELONIN	ANNTISLENKWKLSFQIRTSANGMFSEAVELELANGKKYYVTAVDQVK	237
RTA	PIIALMVYRCAPPPSSOF	267
GELONIN	PKIALLKFDKDPK	251

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FIG.

RTA	I-----FPKQYPIINFTTAGATVQSYTNFIRAVRGRLLTTGADV RHEIPV	44
BRIP	AAKMAKNVDKPLFTATFNVOASSAD-YATFIAGIRNKLRNPAHFSHNRPV	49
RTA	LPN-RVGLPINORFILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFF	93
BRIP	LPPVEPNVPPSRWFHVVLKASPTSAGLT LAIRADNIYLEGFKSSDGTME	99
RTA	HPDNOEDAEATHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNP	143
BRIP	LTPGLIPGATYV-----GFGGTYRDLLGDTDKL-TNVALGRQQ	136
RTA	LEEAI S A L Y-----YYSTGGTQ L P T L A R S F I I C I O M I S E A A R F Q-----Y I E	185
BRIP	LADAVTALHGR TKADKASGPKQQQAREAVTTLVLMVNEATRFQTVSGFVA	186
RTA	GEMRTRIRYNRRSAPDPSVITLENSWGR LSTA IQESNOGAFASPIQLORR	235
BRIP	GLLHPKAVEKSKGKIGNEMKAQVNGWODLSAALLKTDVKPPPGKSPAKFA	236
RTA	NGSKFSVYDVSILIP I I A L M V Y R C A P-----PPSSQF	267
BRIP	PIEKMGVRTAEQAANTLGILLFVEVPGG LTVAKALELFHASGGK	280

FIG.

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RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
MOM011	D-----VNFDLSTATAKTYTKFIEDFRATLPFSHKV-YDIPLLYSTIS	42
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVGYNAGNSAYFFHPDNOED	100
MOM011	--DSRRFILLDLTSYAYETISVAIDVTNVYVAVYRTRDVSYFF---KESP	87
RTA	AEAITHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
MOM011	PEAYNILFKGTR-KITLPTYTGNYENLQTAHAKIRENIDLGLPALSSAITT	136
RTA	LYYSTGGTOLPTLARSFIIICIMISEAARFQYIEGEMRTRIRYNRRSAP	200
MOM011	LFYYNQSA-----PSALLVLIQTAEAAARFKYIERHVAKYVATNFK--P	179
RTA	DPSVITLENSWGLSTAI--QESNOGAFASPIQLORRNGSKFSVYDVS--	246
MOM011	NLAIISLENOWSALSQIFLAQNOGGKFRNPVDLIKPTGERFQVTNVDSD	229
RTA	ILIPIIALMVYRCAPPSSQF	267
MOM011	VVKGNIKLLNSRASTADENFITMTLLGESVNV	263

FIG.

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Parameter	Value	Unit
Temperature	25	°C
Pressure	1	atm
Time	10	min
Concentration	0.1	M
Volume	10	ml
Flow rate	1	ml/min
Wavelength	254	nm
Path length	1	cm
Sample	Water	
Reference	Water	
Blank	Water	
Calibration	Linear	
Correlation coefficient	0.999	
Limit of detection	0.01	mg/L
Limit of quantification	0.05	mg/L
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%
Repeatability	100	%
Recovery	100	%
Stability	100	%
Precision	100	%
Accuracy	100	%
Linearity	100	%
Robustness	100	%
Specificity	100	%
Sensitivity	100	%
Reliability	100	%
Reproducibility	100	%

RTA	IFPKQYPIINF	TTAGATVQSYTNF	IRAVRGRLTTGADVRHEIPVLPNRVG	50
LUFFIN	D-----	VRFSLSGSS	TSYSKFIGDLRKALPSNGTVYNLTILLSSASG	43
RTA	LPINQRFILVEL	SNHAELSVTLALDVTNAYVVGYRAGNSAYFFHPDNQED	100	
LUFFIN	---	ASRYTLM	TSNYDGKAITVAVDVSQLYIMGYLVNSTSYFF---NESD	87
RTA	AEAITHLFTDVQ	NRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150	
LUFFIN	AKLASQYVFKG	STIVTLPYSGNYEKLQTAAGKIREKIPLGFPALDSALT	137	
RTA	LYYSTGGTQ	LPTLARSFIICIONISEAARFOYIEGEMRTRIRYNRRSAP	200	
LUFFIN	IFHYDSTAA---	AAAFVLQTTAEASRFKYIEGQIIERI--SKNQVP	180	
RTA	DPSVITLENS-	WGRNSTAIQ--ESNQAFASPIQLORRNGSKFSVYDVSI	247	
LUFFIN	SLATISLENSL	WSALS [*] KOIQLAQTNNGTFTKTPVITDDKQQRVEITNVTS	230	
RTA	LPIIIALMVYR	CAPPSSQF	267	
LUFFIN	KVVTKNIO	LLN [*] YKQNV	248	

FIG.

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RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRRHEIPVLPNRVG	50
TRICHO	D-----VSFRLSGATSSSYGVFISNLRKALPNERKL-YDIPLL--RSS	40
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGVRAGNSAYFFHPDNOED	100
TRICHO	LPGSORYALIHLTNYADETISVAIDVTNVYIMGYRAGDTSYFF--NEASA	88
RTA	AEAITHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
TRICHO	TEAAKYVFKDAMRKVTLPYSGNYERLOTAAGRIRENIPGLPALDSAITT	138
RTA	LYYSTGGTQPLTARSFIICIONISEAARFQYIEGEMRTRIRYNRRSAP	200
TRICHO	LFYYNANSA-----ASALMVLIOSTSEAAARYKFIEQQIGSRV--DKTFLP	181
RTA	DPSVITLENSWGRNSTAIQ--ESNOGAFASPIQLORRNGSKFSVYDV--S	246
TRICHO	SLAIIISLENSWSALSKOIQIASTNNGQFESPVLINAGNQRVTTINVDAG	231
RTA	ILIPIIALMVYRCAPPPSSQF	267
TRICHO	VVTSNIALLLNRNMA	247

RTA	IFPKOYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
MOMOI	D-----VSFRLSGADPRSYGMFIKDLRNALPFREKVYNIPLLLPSVSG	43
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVGYNAGNSAYFFHPDNOED	100
MOMOI	---AGRYLLMHLFNYDGKTIITVAVDVTNVYINGYLADTTSYFFNEPAAEL	90
RTA	AEAITHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
MOMOI	ASQ--YVFRDARRKITLPYSGNYERLQIAAGKPREKIPIGLPALDSAIST	138
RTA	LYYSTGGTOLPTLARSFIICIMISEAARFQYIEGEMRTRIRYNRRSAP	200
MOMOI	LLHYDSTAA-----AGALLVLIQTAAEARFKYIEQQIQERA--YRDEVP	181
RTA	DPSVITLENSWGRSLTAIQ--ESNQGAFASPIQLORRNGSKFSVYDVSIL	248
MOMOI	SLATISLENSWSGLSKQIQLAQGNNGIFRTPIVLVDNKGNRVOITNVTSK	231
RTA	IPIIALMVY-----RCAPPPSSQF	267
MOMOI	VVTSNIQLLLNTRNIAEGDNGDVSTTHGFSST	263

FIG.

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RTA	IFPKQYPIINFITAGATVQSYTNFIRAVRGRLTTGADVHRHEIPVLPNRVG	50
MAP	A-PTLETIASLDLNNPT--TYLSFITNIRTKVADKTE-----OCTIOKIS	42
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVGYRA---GNSAYFFHPDN	97
MAP	KTFTQRYSYIDLIVSSTOKITLAIIDMADLYVLGYSDIANNKGRAFFFKDV	92
RTA	QEDAEATHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEA	147
MAP	TEAVANNFFPGATGTNRIKLTFTGSYGDLEK-NGGLRKDNPLGIFRLENS	141
RTA	ISALYYSTGGTQLPTLARSFIICIMISEAARFQYIEGEMRTRIRYNRR	197
MAP	IVNIY---GKAGDVKKOAKFFLLAIQMVSEAAARFKYI-SDKIPSEKYEE-	186
RTA	SAPDPSVITLENSWGRSLTAIQESNQGAFAFASPIQLORRNGSKFSVYDYSI	247
MAP	VTVDEYNTALENNWAKLSTAVYNSKPSSTTTATKCOLATSPVTISPWFKT	236
RTA	LIPIIALMVYRCAPPSSOF	267
MAP	VEEIKLVMGLLKSS	250

RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
PAPS	I-----NTITFDAGNATINKYATFMESLRNEAKDPSLKCYGIPMLPNTNS	45
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSA-----YFFHP	95
PAPS	---TIKYL LVKLOGASLKTITLMLRRNLYVNGYSDPYDNKCRYHIFNDI	92
RTA	DNQEDAEATHLFTDVQNRYT--FAFGGNYDRLEQLAG--NLRENIELGNG	142
PAPS	KGTEYSDVENTLCPSSNPRVAKPINYNGLYPTLEKKAGVTSRNEVOLGIQ	142
RTA	PLEEASALYYSTGGTQPTLARSFIIICIMISEAARFQYIEGEMRTI	192
PAPS	ILSSDIGKI--SGQGSFTEKIEAKFLVAIOMVSEARFKYIENQVKTN-	189
RTA	RYNRRSAPDPSVITLENSWGRLSTAIOESNOGAFASPIQLORRNGSKFSV	242
PAPS	-FNRDFSPNDKVLDEENWGKISTAIHNSKNGALPKPLELKNADGTKWIV	238
RTA	YDVSILIPIIALMVRCAPPSSQF	267
PAPS	LRVDEIKPDVGLLNY--VNGTCOAT	261

[illegible]

RTA	IFPKQYPIINF	TTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
SAP6	V-----TSITLDLVNPTAQOYSSFVDKIRNNVKDPNLKYGGTDI--AVIG		43
RTA	LPINQRFILVELSNHAE	SVTLALDVTNAYVVGYRAGNS-----AYFFHP	95
SAP6	PPSKEKFLRINFQSSRG-TVSLGLKRDNLVYVAYLANDNTNVNRAYYFRS		92
RTA	DNQEDAEAI	THLFTDVONRYTFAFGGNYDRLEQ-----LAGNLRNIELG	140
SAP6	EITSAESTALFPEAT	TANOKALEYTEDQYSIEKNAQITQGDQSRKELGLG	142
RTA	NGPLEE	AISALYYSTGGTQPTLARSFIICIONISEAARFQYIEGEMRT	190
SAP6	IDLLSTSMEAV---	NKKARVVKDEARFLLIAIONTAEAAFRFYIQNLVIK	189
RTA	RIRYNRRSAPDP	SVITLENSWGR	239
SAP6	N--FPNKFENSENKVIOFEVNWKKISTAIYGD	AKNGVFNKDYDFGFGKVRO	237
RTA	FSVYDVSILIP	IIALMVYRCAPPPSSQF	267
SAP6	VKDLOMGLL-----	MYLGKPKSSNEAN	259

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INTERNATIONAL SEARCH REPORT

 Int. national application No.
 PCT/US92/09487

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C07K 3/00; C12P 21/00

US CL : 530/350; 435/69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG One search, sequence search (STIC staff)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Vol. 260, No. 22, issued 05 October 1985, J.M. Lambert et al., "Purified Immunotoxins That Are Reactive with Human Lymphoid Cells", pages 12035-12041; see especially pages 12035-12039.	1-22, 66, 69-71, 73-79, 81
Y	US. A. 4,853,871 (PANTOLIANO et al.) 01 AUGUST 1989; see abstract and column 2, line 61-column 3, line 1.	1-22, 66, 69-71, 73-79, 81
Y	GB. A. 2,216,891 (FARMITALIA) 18 OCTOBER 1989; see Derwent abstract.	1-22, 66, 69-71, 73-79, 81



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be part of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P documents published prior to the international filing date but later than the priority date claimed

T

later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A

document member of the same patent family

Date of the actual completion of the international search

02 FEBRUARY 1993

Date of mailing of the international search report

16 FEB 1993

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)*

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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-22, 65, 66, 69-71, 73-79, 81 drawn to a first product RIP analogs and a first process for making same classified in Class 530, subclass 350 and Class 435, subclass 69.1;
- II. Claims 23-37, 80, 85, 87, 88, 90, 92, 94, 96, 99, 100, 102, 104, 106, 108, 109, 113, 117 drawn to a second product polynucleotides encoding RIP analogs classified in Class 536, subclass 23.1;
- III. Claims 38, 110, 114, 118 drawn to a third product vector classified in Class 435, subclass 320.1;
- IV. Claims 39-45, 72, 86, 111, 112, 115, 116, 119, 120 drawn to a fourth product host cell classified in Class 435, subclass 240.1;
- V. Claims 46-64, 82 drawn to a cytotoxic agent classified in Class 530, subclass 391.1;
- VI. Claims 67, 83 drawn to a process for making a cytotoxic agent classified in Class 530, subclass 402;
- VII. Claims 89, 91, 93, 95, 97, 98, 101, 103, 105, 107 drawn to a sixth product fusion protein classified in Class 530, subclass 402;
- VIII. Claims 69, 84 drawn to a third process treatment classified in Class 514, subclass 2.

The foregoing groups represent multiple distinct inventions having no special technical relationship which an advance over the prior art as seen from the prior art cited in this report for which there is no provision in PCT Rules 13.1 and 13.2.

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US92/09487

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 66, 69-71, 73-79, 81 (TELEPHONE PRACTICE)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.